Claim	Proposal Page Ref.
10.(**currently amended**) A composition comprising	

	a polymerizing agent including a molecular tag covalently bonded to a site on the polymerizing agent and	In this method a single tag that is strategically positioned on a DNA polymerase interacts with a color-coded dNTP. **P3,¶1** The chemical attachment of a fluorescence donor to the engineered polymerase and characterization of the modified enzymes will be carried out in Dr. Tu's lab. **P3,¶2** We envision placing a single tag on the polymerase and a unique tag on each dNTP. **P6,¶1** In the first approach, we envision placing a fluorescence donor on the polymerase (i.e. fluorescein or fluorescein-type molecule) and a fluorescence acceptor with a unique fluorescent tag color on each dNTP (i.e. d-rhodamine or similar molecule). **P10,¶1** In one approach, a fluorescence donor will be attached to the polymerase and four unique fluorescence acceptors will each be attached to a different dNTP. The absorption spectra of the donor and acceptor fluorophores should be sufficiently distinct to allow exclusive (preferably) or preferential excitation of the fluorescence donor attached on the polymerase at a chosen wavelength. The emission of the fluorescence donor should have significant overlap with the absorption spectra of the fluorescence acceptors. The four fluorescence acceptors, in the dNTP-attached forms, should each have a unique fluorescence emission distinguishable from that of the other three. **P17,*Approach I***

	a monomer including a molecular tag that is released upon monomer incorporation,	In this method a single tag that is strategically positioned on a DNA polymerase interacts with a color-coded dNTP. **P3,¶1** Once the optimized enzyme is identified, it will be used to stimulate fluorescence transfer with an incoming dNTP (design of detection equipment, Dr. R. Willson; choices of fluorescent donors and acceptors, Drs. D. Tu and X. Gao; choice of site for labeling dNTP, Drs. Briggs, Hardin, Tu, Gao). **P3,¶2** Dr. Gao's group will design, synthesize, and purify tagged dNTPs (base, sugar, or phosphate labeled). **P4,¶4** We envision placing a single tag on the polymerase and a unique tag on each dNTP. **P6,¶1** In the first approach, we envision placing a fluorescence donor on the polymerase (i.e. fluorescein or fluorescein-type molecule) and a fluorescence acceptor with a unique fluorescent tag color on each dNTP (i.e. d-rhodamine or similar molecule). **P10,¶1** One of the ideas presented in this proposal is to put the fluorescent tag on the γ-phosphate such that, upon base incorporation, the tagged PPi will diffuse away from the protein (i.e. FRET will cease). According to our preliminary modeling studies, and the GTP/protein complex model presented in Figure 4 of Kiefer et al., 1998, there appears to be sufficient room for a tag on the γ-phosphate, without inhibiting incorporation. **P14-15,Last ¶** In one approach, a fluorescence donor will be attached to the polymerase and four unique fluorescence acceptors will each be attached to a different dNTP. The absorption spectra of the donor and acceptor fluorophores should be sufficiently distinct to allow exclusive (preferably) or preferential excitation of the fluorescence donor attached on the polymerase at a chosen wavelength. The emission of the fluorescence donor should have significant overlap with the absorption spectra of the fluorescence acceptors. The four fluorescence acceptors, in the dNTP-attached forms, should each have a unique fluorescence emission distinguishable from that of the other three. **P17,*Approach I*** **P20,*Scheme 3***

	where at least one of the tags has a fluorescence property that undergoes a change before, during and/or after each of a sequence of monomer incorporations due to an interaction between the polymerizing agent ~~tag~~ and the monomer ~~tag~~ and	As the correct dNTP is incorporated during the polymerization reaction, the identity of the base is indicated by a signature fluorescent signal. **P3,¶1** Once the optimized enzyme is identified, it will be used to stimulate fluorescence transfer with an incoming dNTP (design of detection equipment, Dr. R. Willson; choices of fluorescent donors and acceptors, Drs. D. Tu and X. Gao; choice of site for labeling dNTP, Drs. Briggs, Hardin, Tu, Gao). **P3,¶2** Dr. Briggs' effort includes identification of candidate amino acids for targeted mutagenesis of the polymerase via modeling of the complex between the (labeled) dNTP and the (labeled) protein. The efficiency of the fluorescence resonance energy transfer (FRET) will be predicted. Dr. Briggs' group will also work closely with Dr. Hardin's group to create the base identification software. **P4,¶5** We envision placing a single tag on the polymerase and a unique tag on each dNTP. As a tagged dNTP is incorporated into the DNA polymer, a characteristic fluorescent signal is emitted that indicates base identity (emission wavelength and/or strength provide signature for base identity). **P6,¶1** Additionally, we will determine whether dNTPs containing tags attached to the terminal (gamma) phosphate are directly detected upon incorporation (four color, base-specific phosphate cleavage stimulates detector). **P6,¶1** As incoming fluorescently-tagged dNTP is bound by the polymerase for DNA elongation, a characteristic fluorescent signal is emitted that indicates base identity (emission wavelength and intensity provide signature for base identity). **P10,¶1** Additionally, we will determine whether dNTPs containing fluorescent tags attached to the terminal (gamma) phosphate are directly detected upon incorporation (four color, base-specific phosphate cleavage stimulates detector). **P10,¶1**

		Mutated and labeled polymerases will be built and energy minimized in a full solvent environment to estimate the effect on the structure of the mutation and/or labeling. This will also provide an estimate of the orientation of the fluorescent label with respect to the dNTP-binding pocket, thereby allowing us to estimate the FRET efficiency prior to measurement. **P13,¶3** In one approach, a fluorescence donor will be attached to the polymerase and four unique fluorescence acceptors will each be attached to a different dNTP. The absorption spectra of the donor and acceptor fluorophores should be sufficiently distinct to allow exclusive (preferably) or preferential excitation of the fluorescence donor attached on the polymerase at a chosen wavelength. The emission of the fluorescence donor should have significant overlap with the absorption spectra of the fluorescence acceptors. The four fluorescence acceptors, in the dNTP-attached forms, should each have a unique fluorescence emission distinguishable from that of the other three. **P17,*Approach I***
		Several sites on dNTPs will be explored for the attachment of the fluorescence acceptors. The initial efforts will be directed to the tagging of the terminal phosphate of dNTP. This approach has a unique advantage. When the incoming, tagged dNTP is bound to the active site of the polymerase, significant FRET from the donor on the polymerase to the acceptor on the dNTP is expected to occur. The unique fluorescence of the acceptor then enables the determination of the identity of the dNTP. Once the tagged dNTP is processed for covalent attachment to the nascent DNA chain, the fluorescence acceptor remains attached to the pyrophosphate and will be released to the medium. In fact, the growing nascent DNA chain will contain only the normal dNMP building units and no fluorescence acceptor molecules at all. In essence, FRET will only occur between the donor on the polymerase and incoming acceptor-labeled dNTP, one at a time. This approach is better than the alternative attachment of the acceptor to any site within the dNMP moiety of the initial dNTPs. In this latter case, the nascent DNA chain will contain multiple molecules of the fluorescence acceptors. Interference with the polymerase reaction and FRET measurements could occur. **P17-18,*Approach I***

		The second approach is to only label the polymerase with a fluorophore. The dNTPs will each be labeled with a quencher of the fluorophore. Ideally, each quencher, when brought to close vicinity to the fluorophore, should have a unique quenching efficiency distinguishable from those by the other quenchers. Therefore, the degrees of quenching will allow the determination of each incoming, labeled dNTP. Four quenchers with distinguishable degrees of efficiencies may not be easy to obtain. Even with only two suitable quenchers, one can label two of the four types of dNTP with the quenchers for one run of the reaction and repeat the same DNA polymerization reaction several times, each time with a different pair of the labeled dNTPs. Results, when taken together, will enable us to definitively determine the complete sequence of the DNA molecule. One obvious advantage of this approach is that fluorescence emission will be coming from a single source. Background noise will be negligible. **P18,*Approach 2***
	where the polymerizing agent lacks the ability to remove a previously incorporated monomer.	Taq DNA polymerase lacks a 3' to 5' exonuclease activity (proofreading activity). This is important for our studies since we do not want the enzyme to remove a base for which fluorescent signal was detected. If the enzyme used in single-molecule DNA sequencing possessed a 3' to 5' exonuclease activity, the enzyme would add another base to replace of the one that had been removed. This newly added base would produce a signature fluorescent signal that would suggest the presence of two identical bases in the template. This type of artifact could be detrimental to the technology. **P12,¶3**
Please note that polymerizing agent and monomer are broader terms than polymerases and dNTPs, respectively, but include polymerases and dNTPs.		
13.(**previously presented**) The composition of claim 10,		

	wherein the polymerizing agent is a polymerase.	***Note polymerases are disclosed throughout proposal*** In this method a single tag that is strategically positioned on a DNA polymerase interacts with a color-coded dNTP. P3,¶1 The chemical attachment of a fluorescence donor to the engineered polymerase and characterization of the modified enzymes will be carried out in Dr. Tu's lab. **P3,¶2** We envision placing a single tag on the polymerase and a unique tag on each dNTP. P6,¶1 **P10,*Enzyme Choice*** through paragraph bridging **P14&15**
16.(**currently amended**) The composition of claim 10,		

	wherein each of the monomers comprises a deoxynucleotide triphosphate (dNTP) and the monomer tag is covalently bonded either directly or through a linker to the ~~β and/or γ phosphate group~~ pyrophosphate moiety of each dNTP.	In this method a single tag that is strategically positioned on a DNA polymerase interacts with a color-coded dNTP. **P3,¶1** Once the optimized enzyme is identified, it will be used to stimulate fluorescence transfer with an incoming dNTP (design of detection equipment, Dr. R. Willson; choices of fluorescent donors and acceptors, Drs. D. Tu and X. Gao; choice of site for labeling dNTP, Drs. Briggs, Hardin, Tu, Gao). **P3,¶2** Dr. Gao's group will design, synthesize, and purify tagged dNTPs (base, sugar, or phosphate labeled). **P4,¶4** We envision placing a single tag on the polymerase and a unique tag on each dNTP. **P6,¶1** In the first approach, we envision placing a fluorescence donor on the polymerase (i.e. fluorescein or fluorescein-type molecule) and a fluorescence acceptor with a unique fluorescent tag color on each dNTP (i.e. d-rhodamine or similar molecule). **P10,¶1** One of the ideas presented in this proposal is to put the fluorescent tag on the γ-phosphate such that, upon base incorporation, the tagged PPi will diffuse away from the protein (i.e. FRET will cease). According to our preliminary modeling studies, and the GTP/protein complex model presented in Figure 4 of Kiefer et al., 1998, there appears to be sufficient room for a tag on the γ-phosphate, without inhibiting incorporation. **P14-15,Last ¶** In one approach, a fluorescence donor will be attached to the polymerase and four unique fluorescence acceptors will each be attached to a different dNTP. The absorption spectra of the donor and acceptor fluorophores should be sufficiently distinct to allow exclusive (preferably) or preferential excitation of the fluorescence donor attached on the polymerase at a chosen wavelength. The emission of the fluorescence donor should have significant overlap with the absorption spectra of the fluorescence acceptors. The four fluorescence acceptors, in the dNTP-attached forms, should each have a unique fluorescence emission distinguishable from that of the other three. P17,Approach I **P20,*Scheme 3***
17.(**currently amended**)	The composition of claim 10,	

	wherein the ~~tags~~ at least one tag comprise a fluorescent tag ~~tags~~ and the fluorescence property comprises a duration, an intensity and/or frequency of emitted fluorescent light.	As the correct dNTP is incorporated during the polymerization reaction, the identity of the base is indicated by a signature fluorescent signal. **P3,¶1** As a tagged dNTP is incorporated into the DNA polymer, a characteristic fluorescent signal is emitted that indicates base identity (emission wavelength and/or strength provide signature for base identity). . . . The fluorescent signals produced upon incorporation will be detected and analyzed to determine DNA base sequence. **P6,¶1** As incoming fluorescently-tagged dNTP is bound by the polymerase for DNA elongation, a characteristic fluorescent signal is emitted that indicates base identity (emission wavelength and intensity provide signature for base identity). **P10,¶1** The raw data generated by the detector will represent between one to four time-dependent data streams of fluorescence wavelengths and intensities, one data stream for each fluorescently labeled base (i.e. wavelength) being monitored. **P22,¶1**
18.(**previously presented**) The composition of claim 17,		

	wherein the fluorescence property is fluorescence resonance energy transfer (FRET) where either the monomer tag or the polymerase tag comprises a donor and the other tag comprises an acceptor and where FRET occurs when the two tags are in close proximity.	***Approach I*** The following principles will be guiding our search for appropriate fluorescence dyes for this work. In one approach, a fluorescence donor will be attached to the polymerase and four unique fluorescence acceptors will each be attached to a different dNTP. The absorption spectra of the donor and acceptor fluorophores should be sufficiently distinct to allow exclusive (preferably) or preferential excitation of the fluorescence donor attached on the polymerase at a chosen wavelength. The emission of the fluorescence donor should have significant overlap with the absorption spectra of the fluorescence acceptors. The four fluorescence acceptors, in the dNTP-attached forms, should each have a unique fluorescence emission distinguishable from that of the other three. Several sites on dNTPs will be explored for the attachment of the fluorescence acceptors. The initial efforts will be directed to the tagging of the terminal phosphate of dNTP. This approach has a unique advantage. When the incoming, tagged dNTP is bound to the active site of the polymerase, significant FRET from the donor on the polymerase to the acceptor on the dNTP is expected to occur. The unique fluorescence of the acceptor then enables the determination of the identity of the dNTP. Once the tagged dNTP is processed for covalent attachment to the nascent DNA chain, the fluorescence acceptor remains attached to the pyrophosphate and will be released to the medium. In fact, the growing nascent DNA chain will contain only the normal dNMP building units and no fluorescence acceptor molecules at all. In essence, FRET will only occur between the donor on the polymerase and incoming acceptor-labeled dNTP, one at a time. This approach is better than the alternative attachment of the acceptor to any site within the dNMP moiety of the initial dNTPs. In this latter case, the nascent DNA chain will contain multiple molecules of the fluorescence acceptors. Interference with the polymerase reaction and FRET measurements could occur. P17,¶3&4 **P15,*Prediction of FRET efficiency***
19.(**previously presented**) The composition of claim 13,		

	wherein the polymerase comprises *Taq* DNA polymerase I having a tag attached to an amino acid at a specific amino acid position of the *Taq* DNA polymerase I, where the amino acid position is selected from the group consisting of 513-518, 643, 647, 649 and 653-661 of SEQ. ID No. 11, where the tag comprises a fluorescent molecule.	***Note: Only in Non-provisional; however, provisional fully disclosed the mechanism by which these sites were selected.*** The identification of sites in the polymerase that are not in contact with other proteins, that should not alter the conformation or folding of the protein, and that are not involved in the function of the protein, will be accomplished by a combination of sequence analyses and molecular docking studies. Regions of the protein surface that are not important for function can be identified, indirectly, by investigating the variation in sequence as a function of evolutionary time and protein function, with use of the evolutionary trace method (Lichtarge et al., 1996). In this approach, amino acid residues that are important for structure or function are found by comparing evolutionary mutations and structural homologies. The polymerases are ideal systems for this type of study, as there are many crystal and co-crystal structures and many available sequences. We will exclude the regions of structural/functional importance from consideration as sites for mutation/labeling. In addition, visual inspection and overlays of available structures in different conformational states, as already available from crystallographic studies, will further assist in identifying areas near the binding site for dNTPs that might be available for mutation and labeling. We envision choosing amino acids somewhat internally located, perhaps surrounding the enzyme active site, to reduce background (i.e. enzyme interacting with non-specifically associated dNTPs). Mutated and labeled polymerases will be built and energy minimized in a full solvent environment to estimate the effect on the structure of the mutation and/or labeling. This will also provide an estimate of the orientation of the fluorescent label with respect to the dNTP-binding pocket, thereby allowing us to estimate the FRET efficiency prior to measurement. **P13,¶3**
50.(**currently amended**)	A composition comprising	

	a polymerizing agent including a molecular tag covalently bonded to a site on the polymerizing agent and	In this method a single tag that is strategically positioned on a DNA polymerase interacts with a color-coded dNTP. **P3,¶1** The chemical attachment of a fluorescence donor to the engineered polymerase and characterization of the modified enzymes will be carried out in Dr. Tu's lab. **P3,¶2** We envision placing a single tag on the polymerase and a unique tag on each dNTP. **P6,¶1** In the first approach, we envision placing a fluorescence donor on the polymerase (i.e. fluorescein or fluorescein-type molecule) and a fluorescence acceptor with a unique fluorescent tag color on each dNTP (i.e. d-rhodamine or similar molecule). **P10,¶1** In one approach, a fluorescence donor will be attached to the polymerase and four unique fluorescence acceptors will each be attached to a different dNTP. The absorption spectra of the donor and acceptor fluorophores should be sufficiently distinct to allow exclusive (preferably) or preferential excitation of the fluorescence donor attached on the polymerase at a chosen wavelength. The emission of the fluorescence donor should have significant overlap with the absorption spectra of the fluorescence acceptors. The four fluorescence acceptors, in the dNTP-attached forms, should each have a unique fluorescence emission distinguishable from that of the other three. **P17,*Approach I***

	a deoxynucleotide triphosphate (dNTP) including a molecular tag covalently bonded directly or through a linker to the ~~β and/or γ phosphate group~~ pyrophosphate moiety of the dNTP,	In this method a single tag that is strategically positioned on a DNA polymerase interacts with a color-coded dNTP. **P3,¶1** Once the optimized enzyme is identified, it will be used to stimulate fluorescence transfer with an incoming dNTP (design of detection equipment, Dr. R. Willson; choices of fluorescent donors and acceptors, Drs. D. Tu and X. Gao; choice of site for labeling dNTP, Drs. Briggs, Hardin, Tu, Gao). **P3,¶2** Dr. Gao's group will design, synthesize, and purify tagged dNTPs (base, sugar, or phosphate labeled). **P4,¶4** We envision placing a single tag on the polymerase and a unique tag on each dNTP. **P6,¶1** In the first approach, we envision placing a fluorescence donor on the polymerase (i.e. fluorescein or fluorescein-type molecule) and a fluorescence acceptor with a unique fluorescent tag color on each dNTP (i.e. d-rhodamine or similar molecule). **P10,¶1** One of the ideas presented in this proposal is to put the fluorescent tag on the γ-phosphate such that, upon base incorporation, the tagged PPi will diffuse away from the protein (i.e. FRET will cease). According to our preliminary modeling studies, and the GTP/protein complex model presented in Figure 4 of Kiefer et al., 1998, there appears to be sufficient room for a tag on the γ-phosphate, without inhibiting incorporation. **P14-15,Last ¶** In one approach, a fluorescence donor will be attached to the polymerase and four unique fluorescence acceptors will each be attached to a different dNTP. The absorption spectra of the donor and acceptor fluorophores should be sufficiently distinct to allow exclusive (preferably) or preferential excitation of the fluorescence donor attached on the polymerase at a chosen wavelength. The emission of the fluorescence donor should have significant overlap with the absorption spectra of the fluorescence acceptors. The four fluorescence acceptors, in the dNTP-attached forms, should each have a unique fluorescence emission distinguishable from that of the other three. **P17,*Approach I*** **P20, *Scheme 3***

	where at least one of the tags has a fluorescence property that undergoes a change before, during and/or after each of a sequence of monomer incorporations due to an interaction between the polymerizing agent ~~tag~~ and the dNTP ~~tag~~.	As the correct dNTP is incorporated during the polymerization reaction, the identity of the base is indicated by a signature fluorescent signal. **P3,¶1** Once the optimized enzyme is identified, it will be used to stimulate fluorescence transfer with an incoming dNTP (design of detection equipment, Dr. R. Willson; choices of fluorescent donors and acceptors, Drs. D. Tu and X. Gao; choice of site for labeling dNTP, Drs. Briggs, Hardin, Tu, Gao). **P3,¶2** Dr. Briggs' effort includes identification of candidate amino acids for targeted mutagenesis of the polymerase via modeling of the complex between the (labeled) dNTP and the (labeled) protein. The efficiency of the fluorescence resonance energy transfer (FRET) will be predicted. Dr. Briggs' group will also work closely with Dr. Hardin's group to create the base identification software. **P4,¶5** We envision placing a single tag on the polymerase and a unique tag on each dNTP. As a tagged dNTP is incorporated into the DNA polymer, a characteristic fluorescent signal is emitted that indicates base identity (emission wavelength and/or strength provide signature for base identity). **P6,¶1** Additionally, we will determine whether dNTPs containing tags attached to the terminal (gamma) phosphate are directly detected upon incorporation (four color, base-specific phosphate cleavage stimulates detector). **P6,¶1** As incoming fluorescently-tagged dNTP is bound by the polymerase for DNA elongation, a characteristic fluorescent signal is emitted that indicates base identity (emission wavelength and intensity provide signature for base identity). **P10,¶1** Additionally, we will determine whether dNTPs containing fluorescent tags attached to the terminal (gamma) phosphate are directly detected upon incorporation (four color, base-specific phosphate cleavage stimulates detector). **P10,¶1**

		Mutated and labeled polymerases will be built and energy minimized in a full solvent environment to estimate the effect on the structure of the mutation and/or labeling. This will also provide an estimate of the orientation of the fluorescent label with respect to the dNTP-binding pocket, thereby allowing us to estimate the FRET efficiency prior to measurement. **P13,¶3** In one approach, a fluorescence donor will be attached to the polymerase and four unique fluorescence acceptors will each be attached to a different dNTP. The absorption spectra of the donor and acceptor fluorophores should be sufficiently distinct to allow exclusive (preferably) or preferential excitation of the fluorescence donor attached on the polymerase at a chosen wavelength. The emission of the fluorescence donor should have significant overlap with the absorption spectra of the fluorescence acceptors. The four fluorescence acceptors, in the dNTP-attached forms, should each have a unique fluorescence emission distinguishable from that of the other three. **P17,*Approach I***
		Several sites on dNTPs will be explored for the attachment of the fluorescence acceptors. The initial efforts will be directed to the tagging of the terminal phosphate of dNTP. This approach has a unique advantage. When the incoming, tagged dNTP is bound to the active site of the polymerase, significant FRET from the donor on the polymerase to the acceptor on the dNTP is expected to occur. The unique fluorescence of the acceptor then enables the determination of the identity of the dNTP. Once the tagged dNTP is processed for covalent attachment to the nascent DNA chain, the fluorescence acceptor remains attached to the pyrophosphate and will be released to the medium. In fact, the growing nascent DNA chain will contain only the normal dNMP building units and no fluorescence acceptor molecules at all. In essence, FRET will only occur between the donor on the polymerase and incoming acceptor-labeled dNTP, one at a time. This approach is better than the alternative attachment of the acceptor to any site within the dNMP moiety of the initial dNTPs. In this latter case, the nascent DNA chain will contain multiple molecules of the fluorescence acceptors. Interference with the polymerase reaction and FRET measurements could occur. **P17-18,*Approach I***

		The second approach is to only label the polymerase with a fluorophore. The dNTPs will each be labeled with a quencher of the fluorophore. Ideally, each quencher, when brought to close vicinity to the fluorophore, should have a unique quenching efficiency distinguishable from those by the other quenchers. Therefore, the degrees of quenching will allow the determination of each incoming, labeled dNTP. Four quenchers with distinguishable degrees of efficiencies may not be easy to obtain. Even with only two suitable quenchers, one can label two of the four types of dNTP with the quenchers for one run of the reaction and repeat the same DNA polymerization reaction several times, each time with a different pair of the labeled dNTPs. Results, when taken together, will enable us to definitively determine the complete sequence of the DNA molecule. One obvious advantage of this approach is that fluorescence emission will be coming from a single source. Background noise will be negligible. **P18,*Approach 2***
51.(**previously presented**) The composition of claim 50,		
	wherein the polymerizing agent is a polymerase or reverse transcriptase.	***Note polymerases are disclosed throughout proposal*** In this method a single tag that is strategically positioned on a DNA polymerase interacts with a color-coded dNTP. **P3,¶1** The chemical attachment of a fluorescence donor to the engineered polymerase and characterization of the modified enzymes will be carried out in Dr. Tu's lab. **P3,¶2** We envision placing a single tag on the polymerase and a unique tag on each dNTP. **P6,¶1** P10,Enzyme Choice through paragraph bridging **P14&15** ***Note: reverse transcriptases were not disclosed, but are a type of polymerase***
52.(**previously presented**) The composition of claim 51,		

	wherein the polymerase is selected from the group consisting of *Taq* DNA polymerase I, T7 DNA polymerase, Sequenase, and the Klenow fragment from *E. coli* DNA polymerase I.	Although crystal structures are available and the enzyme does not contain naturally occurring cysteines, native Taq DNA polymerase is not optimally suited for our purposes since it is not a very processive polymerase (50-80 nucleotides are incorporated before dissociation). It can, however, be appropriately engineered. Specifically, development of a single-molecule DNA sequencer will benefit by using a DNA polymerase that remains associated with the DNA template during the extension phase of the sequencing reaction. Using a highly processive enzyme is expected to minimize complications that may arise from dissociation from the template, which will alter the polymerization rate. However, these rate differences could be compensated for by appropriately modifying the base calling software. Thus, lack of processivity may not limit the sequence lengths achievable by this invention. This feature - processivity - of the native Taq enzyme could negatively impact sequencing run lengths. However, enzymes responsible for replicating the genome are very processive and are able to replicate thousands of bases before dissociating from the template (Kornberg and Baker, 1992). In fact, eukaryotic and prokaryotic DNA polymerases possess mechanisms to overcoming this shortcoming: Increased processivity is achieved through the use of accessory factors (Kelman et al., 1998). A particularly relevant example involves T7 DNA polymerase and its interaction with thioredoxin, a 12 kDa protein produced by E. coli. These proteins associate to form a complex that effectively encircles the DNA template, anchoring the replication complex to the template and achieving a several thousand-fold increase in processivity of T7 DNA polymerase (Tabor et al., 1987; Huber et al., 1987). Processivity can also be altered through genetic engineering, as was elegantly demonstrated using the Klenow fragment from E. coli DNA polymerase I, a polymerase with even lower processivity than Taq. Increased processivity was obtained by introducing the 76 amino acid `processivity domain' from T7 DNA polymerase into the Klenow fragment (Bedford et al., 1997; Bedford et al., 1999). More specifically, this processivity domain contains the thioredoxin binding domain (TBD) from T7 DNA polymerase and it was engineered into the Klenow fragment between the H and H, helices (at the tip of 'thumb' region within the polymerase). This sequence addition caused a thioredoxin-dependent increase in both the processivity and specific activity of Klenow fragment. Thus, we propose to introduce this same region of T7 DNA polymerase into the homologous site of Taq DNA polymerase (Bedford et al., 1999). If necessary, the TBD and thioredoxin can be altered to become more heat stable. **P11,¶3-5**

		Taq DNA polymerase lacks a 3' to 5' exonuclease activity (proofreading activity). This is important for our studies since we do not want the enzyme to remove a base for which fluorescent signal was detected. If the enzyme used in single-molecule DNA sequencing possessed a 3' to 5' exonuclease activity, the enzyme would add another base to replace of the one that had been removed. This newly added base would produce a signature fluorescent signal that would suggest the presence of two identical bases in the template. This type of artifact could be detrimental to the technology. **P12,¶3**
53.(**previously presented**) The composition of claim 51,		
	wherein the reverse transcriptase comprises HIV-1 reverse transcriptase.	***Not disclosed in the proposal, but this enzyme is a type of polymerase.***
54.(**currently amended**) The composition of claim 50,		
	wherein <u>at least one of</u> the tags comprise <u>a</u> fluorescent <u>tag</u> ~~tags~~ and the fluorescence property comprises a duration, an intensity and/or frequency of emitted fluorescent light.	As the correct dNTP is incorporated during the polymerization reaction, the identity of the base is indicated by a signature fluorescent signal. **P3,¶1** As a tagged dNTP is incorporated into the DNA polymer, a characteristic fluorescent signal is emitted that indicates base identity (emission wavelength and/or strength provide signature for base identity). . . . The fluorescent signals produced upon incorporation will be detected and analyzed to determine DNA base sequence. **P6,¶1** In the first approach, we envision placing a fluorescence donor on the polymerase (i.e. fluorescein or fluorescein-type molecule) and a fluorescence acceptor with a unique fluorescent tag color on each dNTP (i.e. d-rhodamine or similar molecule). . . . As incoming fluorescently-tagged dNTP is bound by the polymerase for DNA elongation, a characteristic fluorescent signal is emitted that indicates base identity (emission wavelength and intensity provide signature for base identity). **P10,¶1** The raw data generated by the detector will represent between one to four time-dependent data streams of fluorescence wavelengths and intensities, one data stream for each fluorescently labeled base (i.e. wavelength) being monitored. **P22,¶1**

55.(**previously presented**) The composition of claim 54,		
	wherein the fluorescence property is fluorescence resonance energy transfer (FRET) where either the monomer tag or the polymerase tag comprises a donor and the other tag comprises an acceptor and where FRET occurs when the two tags are in close proximity.	***Approach I*** The following principles will be guiding our search for appropriate fluorescence dyes for this work. In one approach, a fluorescence donor will be attached to the polymerase and four unique fluorescence acceptors will each be attached to a different dNTP. The absorption spectra of the donor and acceptor fluorophores should be sufficiently distinct to allow exclusive (preferably) or preferential excitation of the fluorescence donor attached on the polymerase at a chosen wavelength. The emission of the fluorescence donor should have significant overlap with the absorption spectra of the fluorescence acceptors. The four fluorescence acceptors, in the dNTP-attached forms, should each have a unique fluorescence emission distinguishable from that of the other three. Several sites on dNTPs will be explored for the attachment of the fluorescence acceptors. The initial efforts will be directed to the tagging of the terminal phosphate of dNTP. This approach has a unique advantage. When the incoming, tagged dNTP is bound to the active site of the polymerase, significant FRET from the donor on the polymerase to the acceptor on the dNTP is expected to occur. The unique fluorescence of the acceptor then enables the determination of the identity of the dNTP. Once the tagged dNTP is processed for covalent attachment to the nascent DNA chain, the fluorescence acceptor remains attached to the pyrophosphate and will be released to the medium. In fact, the growing nascent DNA chain will contain only the normal dNMP building units and no fluorescence acceptor molecules at all. In essence, FRET will only occur between the donor on the polymerase and incoming acceptor-labeled dNTP, one at a time. This approach is better than the alternative attachment of the acceptor to any site within the dNMP moiety of the initial dNTPs. In this latter case, the nascent DNA chain will contain multiple molecules of the fluorescence acceptors. Interference with the polymerase reaction and FRET measurements could occur. **P17,¶3&4** **P15,*Prediction of FRET efficiency***

56.(**previously presented**) The composition of claim 52,	
wherein the polymerase comprises *Taq* DNA polymerase I having a tag attached to an amino acid at a specific amino acid position of the *Taq* DNA polymerase I, where the amino acid position is selected from the group consisting of 513-518, 643, 647, 649 and 653-661 of SEQ. ID No. 11, where the tag comprises a fluorescent molecule.	***Note: Only in Non-provisional; however, provisional fully disclosed the mechanism by which these sites were selected.*** The identification of sites in the polymerase that are not in contact with other proteins, that should not alter the conformation or folding of the protein, and that are not involved in the function of the protein, will be accomplished by a combination of sequence analyses and molecular docking studies. Regions of the protein surface that are not important for function can be identified, indirectly, by investigating the variation in sequence as a function of evolutionary time and protein function, with use of the evolutionary trace method (Lichtarge et al., 1996). In this approach, amino acid residues that are important for structure or function are found by comparing evolutionary mutations and structural homologies. The polymerases are ideal systems for this type of study, as there are many crystal and co-crystal structures and many available sequences. We will exclude the regions of structural/functional importance from consideration as sites for mutation/labeling. In addition, visual inspection and overlays of available structures in different conformational states, as already available from crystallographic studies, will further assist in identifying areas near the binding site for dNTPs that might be available for mutation and labeling. We envision choosing amino acids somewhat internally located, perhaps surrounding the enzyme active site, to reduce background (i.e. enzyme interacting with non-specifically associated dNTPs). Mutated and labeled polymerases will be built and energy minimized in a full solvent environment to estimate the effect on the structure of the mutation and/or labeling. This will also provide an estimate of the orientation of the fluorescent label with respect to the dNTP-binding pocket, thereby allowing us to estimate the FRET efficiency prior to measurement. **P13,¶3**
64.(**currently amended**) A composition comprising	

	a polymerizing agent including a molecular tag covalently bonded to a site on the polymerizing agent and	In this method a single tag that is strategically positioned on a DNA polymerase interacts with a color-coded dNTP. **P3,¶1** The chemical attachment of a fluorescence donor to the engineered polymerase and characterization of the modified enzymes will be carried out in Dr. Tu's lab. **P3,¶2** We envision placing a single tag on the polymerase and a unique tag on each dNTP. **P6,¶1** In the first approach, we envision placing a fluorescence donor on the polymerase (i.e. fluorescein or fluorescein-type molecule) and a fluorescence acceptor with a unique fluorescent tag color on each dNTP (i.e. d-rhodamine or similar molecule). **P10,¶1** In one approach, a fluorescence donor will be attached to the polymerase and four unique fluorescence acceptors will each be attached to a different dNTP. The absorption spectra of the donor and acceptor fluorophores should be sufficiently distinct to allow exclusive (preferably) or preferential excitation of the fluorescence donor attached on the polymerase at a chosen wavelength. The emission of the fluorescence donor should have significant overlap with the absorption spectra of the fluorescence acceptors. The four fluorescence acceptors, in the dNTP-attached forms, should each have a unique fluorescence emission distinguishable from that of the other three. **P17,*Approach I***

	a deoxynucleotide triphosphate (dNTP) including a molecular tag covalently bonded directly or through a linker to the γ phosphate group of the dNTP,	In this method a single tag that is strategically positioned on a DNA polymerase interacts with a color-coded dNTP. **P3,¶1** Once the optimized enzyme is identified, it will be used to stimulate fluorescence transfer with an incoming dNTP (design of detection equipment, Dr. R. Willson; choices of fluorescent donors and acceptors, Drs. D. Tu and X. Gao; choice of site for labeling dNTP, Drs. Briggs, Hardin, Tu, Gao). **P3,¶2** Dr. Gao's group will design, synthesize, and purify tagged dNTPs (base, sugar, or phosphate labeled). **P4,¶4** We envision placing a single tag on the polymerase and a unique tag on each dNTP. **P6,¶1** In the first approach, we envision placing a fluorescence donor on the polymerase (i.e. fluorescein or fluorescein-type molecule) and a fluorescence acceptor with a unique fluorescent tag color on each dNTP (i.e. d-rhodamine or similar molecule). **P10,¶1** One of the ideas presented in this proposal is to put the fluorescent tag on the γ-phosphate such that, upon base incorporation, the tagged PPi will diffuse away from the protein (i.e. FRET will cease). According to our preliminary modeling studies, and the GTP/protein complex model presented in Figure 4 of Kiefer et al., 1998, there appears to be sufficient room for a tag on the γ-phosphate, without inhibiting incorporation. **P14-15,Last ¶** In one approach, a fluorescence donor will be attached to the polymerase and four unique fluorescence acceptors will each be attached to a different dNTP. The absorption spectra of the donor and acceptor fluorophores should be sufficiently distinct to allow exclusive (preferably) or preferential excitation of the fluorescence donor attached on the polymerase at a chosen wavelength. The emission of the fluorescence donor should have significant overlap with the absorption spectra of the fluorescence acceptors. The four fluorescence acceptors, in the dNTP-attached forms, should each have a unique fluorescence emission distinguishable from that of the other three. P17,Approach I **P20, *Scheme 3***

	where at least one of the tags has a fluorescence property that undergoes a change before, during and/or after each of a sequence of monomer incorporations due to an interaction between the polymerizing agent ~~tag~~ and the dNTP ~~tag~~.	As the correct dNTP is incorporated during the polymerization reaction, the identity of the base is indicated by a signature fluorescent signal. **P3,¶1** Once the optimized enzyme is identified, it will be used to stimulate fluorescence transfer with an incoming dNTP (design of detection equipment, Dr. R. Willson; choices of fluorescent donors and acceptors, Drs. D. Tu and X. Gao; choice of site for labeling dNTP, Drs. Briggs, Hardin, Tu, Gao). **P3,¶2** Dr. Briggs' effort includes identification of candidate amino acids for targeted mutagenesis of the polymerase via modeling of the complex between the (labeled) dNTP and the (labeled) protein. The efficiency of the fluorescence resonance energy transfer (FRET) will be predicted. Dr. Briggs' group will also work closely with Dr. Hardin's group to create the base identification software. **P4,¶5** We envision placing a single tag on the polymerase and a unique tag on each dNTP. As a tagged dNTP is incorporated into the DNA polymer, a characteristic fluorescent signal is emitted that indicates base identity (emission wavelength and/or strength provide signature for base identity). **P6,¶1** Additionally, we will determine whether dNTPs containing tags attached to the terminal (gamma) phosphate are directly detected upon incorporation (four color, base-specific phosphate cleavage stimulates detector). **P6,¶1** As incoming fluorescently-tagged dNTP is bound by the polymerase for DNA elongation, a characteristic fluorescent signal is emitted that indicates base identity (emission wavelength and intensity provide signature for base identity). **P10,¶1** Additionally, we will determine whether dNTPs containing fluorescent tags attached to the terminal (gamma) phosphate are directly detected upon incorporation (four color, base-specific phosphate cleavage stimulates detector). **P10,¶1**

		Mutated and labeled polymerases will be built and energy minimized in a full solvent environment to estimate the effect on the structure of the mutation and/or labeling. This will also provide an estimate of the orientation of the fluorescent label with respect to the dNTP-binding pocket, thereby allowing us to estimate the FRET efficiency prior to measurement. **P13,¶3** In one approach, a fluorescence donor will be attached to the polymerase and four unique fluorescence acceptors will each be attached to a different dNTP. The absorption spectra of the donor and acceptor fluorophores should be sufficiently distinct to allow exclusive (preferably) or preferential excitation of the fluorescence donor attached on the polymerase at a chosen wavelength. The emission of the fluorescence donor should have significant overlap with the absorption spectra of the fluorescence acceptors. The four fluorescence acceptors, in the dNTP-attached forms, should each have a unique fluorescence emission distinguishable from that of the other three. **P17,*Approach I***
		Several sites on dNTPs will be explored for the attachment of the fluorescence acceptors. The initial efforts will be directed to the tagging of the terminal phosphate of dNTP. This approach has a unique advantage. When the incoming, tagged dNTP is bound to the active site of the polymerase, significant FRET from the donor on the polymerase to the acceptor on the dNTP is expected to occur. The unique fluorescence of the acceptor then enables the determination of the identity of the dNTP. Once the tagged dNTP is processed for covalent attachment to the nascent DNA chain, the fluorescence acceptor remains attached to the pyrophosphate and will be released to the medium. In fact, the growing nascent DNA chain will contain only the normal dNMP building units and no fluorescence acceptor molecules at all. In essence, FRET will only occur between the donor on the polymerase and incoming acceptor-labeled dNTP, one at a time. This approach is better than the alternative attachment of the acceptor to any site within the dNMP moiety of the initial dNTPs. In this latter case, the nascent DNA chain will contain multiple molecules of the fluorescence acceptors. Interference with the polymerase reaction and FRET measurements could occur. **P17-18,*Approach I***

		The second approach is to only label the polymerase with a fluorophore. The dNTPs will each be labeled with a quencher of the fluorophore. Ideally, each quencher, when brought to close vicinity to the fluorophore, should have a unique quenching efficiency distinguishable from those by the other quenchers. Therefore, the degrees of quenching will allow the determination of each incoming, labeled dNTP. Four quenchers with distinguishable degrees of efficiencies may not be easy to obtain. Even with only two suitable quenchers, one can label two of the four types of dNTP with the quenchers for one run of the reaction and repeat the same DNA polymerization reaction several times, each time with a different pair of the labeled dNTPs. Results, when taken together, will enable us to definitively determine the complete sequence of the DNA molecule. One obvious advantage of this approach is that fluorescence emission will be coming from a single source. Background noise will be negligible. **P18,*Approach 2***
65.(**previously presented**) The composition of claim 64,		
	wherein the polymerizing agent is a polymerase or reverse transcriptase.	***Note polymerases are disclosed throughout proposal*** In this method a single tag that is strategically positioned on a DNA polymerase interacts with a color-coded dNTP. **P3,¶1** The chemical attachment of a fluorescence donor to the engineered polymerase and characterization of the modified enzymes will be carried out in Dr. Tu's lab. **P3,¶2** We envision placing a single tag on the polymerase and a unique tag on each dNTP. **P6,¶1** P10,Enzyme Choice through paragraph bridging **P14&15** ***Note: reverse transcriptases were not disclosed, but are a type of polymerase***
66.(**previously presented**) The composition of claim 65,		

	wherein the polymerase is selected from the group consisting of *Taq* DNA polymerase I, T7 DNA polymerase, Sequenase, and the Klenow fragment from *E. coli* DNA polymerase I.	Although crystal structures are available and the enzyme does not contain naturally occurring cysteines, native Taq DNA polymerase is not optimally suited for our purposes since it is not a very processive polymerase (50-80 nucleotides are incorporated before dissociation). It can, however, be appropriately engineered. Specifically, development of a single-molecule DNA sequencer will benefit by using a DNA polymerase that remains associated with the DNA template during the extension phase of the sequencing reaction. Using a highly processive enzyme is expected to minimize complications that may arise from dissociation from the template, which will alter the polymerization rate. However, these rate differences could be compensated for by appropriately modifying the base calling software. Thus, lack of processivity may not limit the sequence lengths achievable by this invention. This feature - processivity - of the native Taq enzyme could negatively impact sequencing run lengths. However, enzymes responsible for replicating the genome are very processive and are able to replicate thousands of bases before dissociating from the template (Kornberg and Baker, 1992). In fact, eukaryotic and prokaryotic DNA polymerases possess mechanisms to overcoming this shortcoming: Increased processivity is achieved through the use of accessory factors (Kelman et al., 1998). A particularly relevant example involves T7 DNA polymerase and its interaction with thioredoxin, a 12 kDa protein produced by E. coli. These proteins associate to form a complex that effectively encircles the DNA template, anchoring the replication complex to the template and achieving a several thousand-fold increase in processivity of T7 DNA polymerase (Tabor et al., 1987; Huber et al., 1987). Processivity can also be altered through genetic engineering, as was elegantly demonstrated using the Klenow fragment from E. coli DNA polymerase I, a polymerase with even lower processivity than Taq. Increased processivity was obtained by introducing the 76 amino acid `processivity domain' from T7 DNA polymerase into the Klenow fragment (Bedford et al., 1997; Bedford et al., 1999). More specifically, this processivity domain contains the thioredoxin binding domain (TBD) from T7 DNA polymerase and it was engineered into the Klenow fragment between the H and H, helices (at the tip of 'thumb' region within the polymerase). This sequence addition caused a thioredoxin-dependent increase in both the processivity and specific activity of Klenow fragment. Thus, we propose to introduce this same region of T7 DNA polymerase into the homologous site of Taq DNA polymerase (Bedford et al., 1999). If necessary, the TBD and thioredoxin can be altered to become more heat stable. **P11,¶3-5**

		Taq DNA polymerase lacks a 3' to 5' exonuclease activity (proofreading activity). This is important for our studies since we do not want the enzyme to remove a base for which fluorescent signal was detected. If the enzyme used in single-molecule DNA sequencing possessed a 3' to 5' exonuclease activity, the enzyme would add another base to replace of the one that had been removed. This newly added base would produce a signature fluorescent signal that would suggest the presence of two identical bases in the template. This type of artifact could be detrimental to the technology. **P12,¶3**
67.(**previously presented**) The composition of claim 65,		
	wherein the reverse transcriptase comprises HIV-1 reverse transcriptase.	***Not disclosed in the proposal, but this enzyme is a type of polymerase.***
68.(**currently amended**) The composition of claim 64,		
	wherein <u>at least one of</u> the tags comprise <u>a</u> fluorescent <u>tag</u> ~~tags~~ and the fluorescence property comprises a duration, an intensity and/or frequency of emitted fluorescent light.	As the correct dNTP is incorporated during the polymerization reaction, the identity of the base is indicated by a signature fluorescent signal. **P3,¶1** As a tagged dNTP is incorporated into the DNA polymer, a characteristic fluorescent signal is emitted that indicates base identity (emission wavelength and/or strength provide signature for base identity). . . . The fluorescent signals produced upon incorporation will be detected and analyzed to determine DNA base sequence. **P6,¶1** In the first approach, we envision placing a fluorescence donor on the polymerase (i.e. fluorescein or fluorescein-type molecule) and a fluorescence acceptor with a unique fluorescent tag color on each dNTP (i.e. d-rhodamine or similar molecule). . . . As incoming fluorescently-tagged dNTP is bound by the polymerase for DNA elongation, a characteristic fluorescent signal is emitted that indicates base identity (emission wavelength and intensity provide signature for base identity). **P10,¶1** The raw data generated by the detector will represent between one to four time-dependent data streams of fluorescence wavelengths and intensities, one data stream for each fluorescently labeled base (i.e. wavelength) being monitored. **P22,¶1**

69.(**previously presented**) The composition of claim 68,		
	wherein the fluorescence property is fluorescence resonance energy transfer (FRET) where either the monomer tag or the polymerase tag comprises a donor and the other tag comprises an acceptor and where FRET occurs when the two tags are in close proximity.	***Approach I*** The following principles will be guiding our search for appropriate fluorescence dyes for this work. In one approach, a fluorescence donor will be attached to the polymerase and four unique fluorescence acceptors will each be attached to a different dNTP. The absorption spectra of the donor and acceptor fluorophores should be sufficiently distinct to allow exclusive (preferably) or preferential excitation of the fluorescence donor attached on the polymerase at a chosen wavelength. The emission of the fluorescence donor should have significant overlap with the absorption spectra of the fluorescence acceptors. The four fluorescence acceptors, in the dNTP-attached forms, should each have a unique fluorescence emission distinguishable from that of the other three. Several sites on dNTPs will be explored for the attachment of the fluorescence acceptors. The initial efforts will be directed to the tagging of the terminal phosphate of dNTP. This approach has a unique advantage. When the incoming, tagged dNTP is bound to the active site of the polymerase, significant FRET from the donor on the polymerase to the acceptor on the dNTP is expected to occur. The unique fluorescence of the acceptor then enables the determination of the identity of the dNTP. Once the tagged dNTP is processed for covalent attachment to the nascent DNA chain, the fluorescence acceptor remains attached to the pyrophosphate and will be released to the medium. In fact, the growing nascent DNA chain will contain only the normal dNMP building units and no fluorescence acceptor molecules at all. In essence, FRET will only occur between the donor on the polymerase and incoming acceptor-labeled dNTP, one at a time. This approach is better than the alternative attachment of the acceptor to any site within the dNMP moiety of the initial dNTPs. In this latter case, the nascent DNA chain will contain multiple molecules of the fluorescence acceptors. Interference with the polymerase reaction and FRET measurements could occur. **P17,¶3&4** **P15,*Prediction of FRET efficiency***

70.(**previously presented**) The composition of claim 66,	
wherein the polymerase comprises *Taq* DNA polymerase I having a tag attached to an amino acid at a specific amino acid position of the *Taq* DNA polymerase I, where the amino acid position is selected from the group consisting of 513-518, 643, 647, 649 and 653-661 of SEQ. ID No. 11, where the tag comprises a fluorescent molecule.	***Note: Only in Non-provisional; however, provisional fully disclosed the mechanism by which these sites were selected.*** The identification of sites in the polymerase that are not in contact with other proteins, that should not alter the conformation or folding of the protein, and that are not involved in the function of the protein, will be accomplished by a combination of sequence analyses and molecular docking studies. Regions of the protein surface that are not important for function can be identified, indirectly, by investigating the variation in sequence as a function of evolutionary time and protein function, with use of the evolutionary trace method (Lichtarge et al., 1996). In this approach, amino acid residues that are important for structure or function are found by comparing evolutionary mutations and structural homologies. The polymerases are ideal systems for this type of study, as there are many crystal and co-crystal structures and many available sequences. We will exclude the regions of structural/functional importance from consideration as sites for mutation/labeling. In addition, visual inspection and overlays of available structures in different conformational states, as already available from crystallographic studies, will further assist in identifying areas near the binding site for dNTPs that might be available for mutation and labeling. We envision choosing amino acids somewhat internally located, perhaps surrounding the enzyme active site, to reduce background (i.e. enzyme interacting with non-specifically associated dNTPs). Mutated and labeled polymerases will be built and energy minimized in a full solvent environment to estimate the effect on the structure of the mutation and/or labeling. This will also provide an estimate of the orientation of the fluorescent label with respect to the dNTP-binding pocket, thereby allowing us to estimate the FRET efficiency prior to measurement. **P13,¶3**
71.(**currently amended**) A composition comprising	

	a polymerizing agent including a molecular tag covalently bonded to a site on the polymerizing agent and	In this method a single tag that is strategically positioned on a DNA polymerase interacts with a color-coded dNTP. **P3,¶1** The chemical attachment of a fluorescence donor to the engineered polymerase and characterization of the modified enzymes will be carried out in Dr. Tu's lab. **P3,¶2** We envision placing a single tag on the polymerase and a unique tag on each dNTP. **P6,¶1** In the first approach, we envision placing a fluorescence donor on the polymerase (i.e. fluorescein or fluorescein-type molecule) and a fluorescence acceptor with a unique fluorescent tag color on each dNTP (i.e. d-rhodamine or similar molecule). **P10,¶1** In one approach, a fluorescence donor will be attached to the polymerase and four unique fluorescence acceptors will each be attached to a different dNTP. The absorption spectra of the donor and acceptor fluorophores should be sufficiently distinct to allow exclusive (preferably) or preferential excitation of the fluorescence donor attached on the polymerase at a chosen wavelength. The emission of the fluorescence donor should have significant overlap with the absorption spectra of the fluorescence acceptors. The four fluorescence acceptors, in the dNTP-attached forms, should each have a unique fluorescence emission distinguishable from that of the other three. **P17,Approach I**

	a monomer including a molecular tag covalently bonded directly or through a linker to the terminal phosphate of the monomer,	In this method a single tag that is strategically positioned on a DNA polymerase interacts with a color-coded dNTP. **P3,¶1** Once the optimized enzyme is identified, it will be used to stimulate fluorescence transfer with an incoming dNTP (design of detection equipment, Dr. R. Willson; choices of fluorescent donors and acceptors, Drs. D. Tu and X. Gao; choice of site for labeling dNTP, Drs. Briggs, Hardin, Tu, Gao). **P3,¶2** Dr. Gao's group will design, synthesize, and purify tagged dNTPs (base, sugar, or phosphate labeled). **P4,¶4** We envision placing a single tag on the polymerase and a unique tag on each dNTP. **P6,¶1** In the first approach, we envision placing a fluorescence donor on the polymerase (i.e. fluorescein or fluorescein-type molecule) and a fluorescence acceptor with a unique fluorescent tag color on each dNTP (i.e. d-rhodamine or similar molecule). **P10,¶1** One of the ideas presented in this proposal is to put the fluorescent tag on the γ-phosphate such that, upon base incorporation, the tagged PPi will diffuse away from the protein (i.e. FRET will cease). According to our preliminary modeling studies, and the GTP/protein complex model presented in Figure 4 of Kiefer et al., 1998, there appears to be sufficient room for a tag on the γ-phosphate, without inhibiting incorporation. **P14-15,Last ¶** In one approach, a fluorescence donor will be attached to the polymerase and four unique fluorescence acceptors will each be attached to a different dNTP. The absorption spectra of the donor and acceptor fluorophores should be sufficiently distinct to allow exclusive (preferably) or preferential excitation of the fluorescence donor attached on the polymerase at a chosen wavelength. The emission of the fluorescence donor should have significant overlap with the absorption spectra of the fluorescence acceptors. The four fluorescence acceptors, in the dNTP-attached forms, should each have a unique fluorescence emission distinguishable from that of the other three. **P17,Approach I** **P20, *Scheme 3***

	where at least one of the tags has a fluorescence property that undergoes a change before, during and/or after each of a sequence of monomer incorporations due to an interaction between the polymerizing agent ~~tag~~ and the monomer ~~tag~~.	As the correct dNTP is incorporated during the polymerization reaction, the identity of the base is indicated by a signature fluorescent signal. **P3,¶1** Once the optimized enzyme is identified, it will be used to stimulate fluorescence transfer with an incoming dNTP (design of detection equipment, Dr. R. Willson; choices of fluorescent donors and acceptors, Drs. D. Tu and X. Gao; choice of site for labeling dNTP, Drs. Briggs, Hardin, Tu, Gao). **P3,¶2** Dr. Briggs' effort includes identification of candidate amino acids for targeted mutagenesis of the polymerase via modeling of the complex between the (labeled) dNTP and the (labeled) protein. The efficiency of the fluorescence resonance energy transfer (FRET) will be predicted. Dr. Briggs' group will also work closely with Dr. Hardin's group to create the base identification software. **P4,¶5** We envision placing a single tag on the polymerase and a unique tag on each dNTP. As a tagged dNTP is incorporated into the DNA polymer, a characteristic fluorescent signal is emitted that indicates base identity (emission wavelength and/or strength provide signature for base identity). **P6,¶1** Additionally, we will determine whether dNTPs containing tags attached to the terminal (gamma) phosphate are directly detected upon incorporation (four color, base-specific phosphate cleavage stimulates detector). **P6,¶1** As incoming fluorescently-tagged dNTP is bound by the polymerase for DNA elongation, a characteristic fluorescent signal is emitted that indicates base identity (emission wavelength and intensity provide signature for base identity). **P10,¶1** Additionally, we will determine whether dNTPs containing fluorescent tags attached to the terminal (gamma) phosphate are directly detected upon incorporation (four color, base-specific phosphate cleavage stimulates detector). **P10,¶1**

		Mutated and labeled polymerases will be built and energy minimized in a full solvent environment to estimate the effect on the structure of the mutation and/or labeling. This will also provide an estimate of the orientation of the fluorescent label with respect to the dNTP-binding pocket, thereby allowing us to estimate the FRET efficiency prior to measurement. **P13,¶3** In one approach, a fluorescence donor will be attached to the polymerase and four unique fluorescence acceptors will each be attached to a different dNTP. The absorption spectra of the donor and acceptor fluorophores should be sufficiently distinct to allow exclusive (preferably) or preferential excitation of the fluorescence donor attached on the polymerase at a chosen wavelength. The emission of the fluorescence donor should have significant overlap with the absorption spectra of the fluorescence acceptors. The four fluorescence acceptors, in the dNTP-attached forms, should each have a unique fluorescence emission distinguishable from that of the other three. **P17,*Approach I***
		Several sites on dNTPs will be explored for the attachment of the fluorescence acceptors. The initial efforts will be directed to the tagging of the terminal phosphate of dNTP. This approach has a unique advantage. When the incoming, tagged dNTP is bound to the active site of the polymerase, significant FRET from the donor on the polymerase to the acceptor on the dNTP is expected to occur. The unique fluorescence of the acceptor then enables the determination of the identity of the dNTP. Once the tagged dNTP is processed for covalent attachment to the nascent DNA chain, the fluorescence acceptor remains attached to the pyrophosphate and will be released to the medium. In fact, the growing nascent DNA chain will contain only the normal dNMP building units and no fluorescence acceptor molecules at all. In essence, FRET will only occur between the donor on the polymerase and incoming acceptor-labeled dNTP, one at a time. This approach is better than the alternative attachment of the acceptor to any site within the dNMP moiety of the initial dNTPs. In this latter case, the nascent DNA chain will contain multiple molecules of the fluorescence acceptors. Interference with the polymerase reaction and FRET measurements could occur. **P17-18,*Approach I***

		The second approach is to only label the polymerase with a fluorophore. The dNTPs will each be labeled with a quencher of the fluorophore. Ideally, each quencher, when brought to close vicinity to the fluorophore, should have a unique quenching efficiency distinguishable from those by the other quenchers. Therefore, the degrees of quenching will allow the determination of each incoming, labeled dNTP. Four quenchers with distinguishable degrees of efficiencies may not be easy to obtain. Even with only two suitable quenchers, one can label two of the four types of dNTP with the quenchers for one run of the reaction and repeat the same DNA polymerization reaction several times, each time with a different pair of the labeled dNTPs. Results, when taken together, will enable us to definitively determine the complete sequence of the DNA molecule. One obvious advantage of this approach is that fluorescence emission will be coming from a single source. Background noise will be negligible. **P18,*Approach 2***
72.(**previously presented**) The composition of claim 71,		
	wherein the polymerizing agent is a polymerase or reverse transcriptase.	***Note polymerases are disclosed throughout proposal*** In this method a single tag that is strategically positioned on a DNA polymerase interacts with a color-coded dNTP. **P3,¶1** The chemical attachment of a fluorescence donor to the engineered polymerase and characterization of the modified enzymes will be carried out in Dr. Tu's lab. P3,¶2 We envision placing a single tag on the polymerase and a unique tag on each dNTP. **P6,¶1** **P10,Enzyme Choice through paragraph bridging P14&15** ***Note: reverse transcriptases were not disclosed, but are a type of polymerase***
73.(**previously presented**) The composition of claim 72,		

	wherein the polymerase is selected from the group consisting of *Taq* DNA polymerase I, T7 DNA polymerase, Sequenase, and the Klenow fragment from *E. coli* DNA polymerase I.	Although crystal structures are available and the enzyme does not contain naturally occurring cysteines, native Taq DNA polymerase is not optimally suited for our purposes since it is not a very processive polymerase (50-80 nucleotides are incorporated before dissociation). It can, however, be appropriately engineered. Specifically, development of a single-molecule DNA sequencer will benefit by using a DNA polymerase that remains associated with the DNA template during the extension phase of the sequencing reaction. Using a highly processive enzyme is expected to minimize complications that may arise from dissociation from the template, which will alter the polymerization rate. However, these rate differences could be compensated for by appropriately modifying the base calling software. Thus, lack of processivity may not limit the sequence lengths achievable by this invention. This feature - processivity - of the native Taq enzyme could negatively impact sequencing run lengths. However, enzymes responsible for replicating the genome are very processive and are able to replicate thousands of bases before dissociating from the template (Kornberg and Baker, 1992). In fact, eukaryotic and prokaryotic DNA polymerases possess mechanisms to overcoming this shortcoming: Increased processivity is achieved through the use of accessory factors (Kelman et al., 1998). A particularly relevant example involves T7 DNA polymerase and its interaction with thioredoxin, a 12 kDa protein produced by E. coli. These proteins associate to form a complex that effectively encircles the DNA template, anchoring the replication complex to the template and achieving a several thousand-fold increase in processivity of T7 DNA polymerase (Tabor et al., 1987; Huber et al., 1987). Processivity can also be altered through genetic engineering, as was elegantly demonstrated using the Klenow fragment from E. coli DNA polymerase I, a polymerase with even lower processivity than Taq. Increased processivity was obtained by introducing the 76 amino acid `processivity domain' from T7 DNA polymerase into the Klenow fragment (Bedford et al., 1997; Bedford et al., 1999). More specifically, this processivity domain contains the thioredoxin binding domain (TBD) from T7 DNA polymerase and it was engineered into the Klenow fragment between the H and H, helices (at the tip of 'thumb' region within the polymerase). This sequence addition caused a thioredoxin-dependent increase in both the processivity and specific activity of Klenow fragment. Thus, we propose to introduce this same region of T7 DNA polymerase into the homologous site of Taq DNA polymerase (Bedford et al., 1999). If necessary, the TBD and thioredoxin can be altered to become more heat stable. **P11,¶3-5**

		Taq DNA polymerase lacks a 3' to 5' exonuclease activity (proofreading activity). This is important for our studies since we do not want the enzyme to remove a base for which fluorescent signal was detected. If the enzyme used in single-molecule DNA sequencing possessed a 3' to 5' exonuclease activity, the enzyme would add another base to replace of the one that had been removed. This newly added base would produce a signature fluorescent signal that would suggest the presence of two identical bases in the template. This type of artifact could be detrimental to the technology. **P12,¶3**
74.(**previously presented**) The composition of claim 72,		
	wherein the reverse transcriptase comprises HIV-1 reverse transcriptase.	***Not disclosed in the proposal, but this enzyme is a type of polymerase.***
76.(**currently amended**) The composition of claim ~~75~~71,		
	wherein at least one of the tags comprises a fluorescent tag ~~tags~~ and the fluorescence property comprises a duration, an intensity and/or frequency of emitted fluorescent light.	As the correct dNTP is incorporated during the polymerization reaction, the identity of the base is indicated by a signature fluorescent signal. **P3,¶1** As a tagged dNTP is incorporated into the DNA polymer, a characteristic fluorescent signal is emitted that indicates base identity (emission wavelength and/or strength provide signature for base identity). . . . The fluorescent signals produced upon incorporation will be detected and analyzed to determine DNA base sequence. **P6,¶1** As incoming fluorescently-tagged dNTP is bound by the polymerase for DNA elongation, a characteristic fluorescent signal is emitted that indicates base identity (emission wavelength and intensity provide signature for base identity). **P10,¶1** The raw data generated by the detector will represent between one to four time-dependent data streams of fluorescence wavelengths and intensities, one data stream for each fluorescently labeled base (i.e. wavelength) being monitored. **P22,¶1**
77.(**previously presented**) The composition of claim 76,		

	wherein the fluorescence property is fluorescence resonance energy transfer (FRET) where either the monomer tag or the polymerase tag comprises a donor and the other tag comprises an acceptor and where FRET occurs when the two tags are in close proximity.	***Approach I*** The following principles will be guiding our search for appropriate fluorescence dyes for this work. In one approach, a fluorescence donor will be attached to the polymerase and four unique fluorescence acceptors will each be attached to a different dNTP. The absorption spectra of the donor and acceptor fluorophores should be sufficiently distinct to allow exclusive (preferably) or preferential excitation of the fluorescence donor attached on the polymerase at a chosen wavelength. The emission of the fluorescence donor should have significant overlap with the absorption spectra of the fluorescence acceptors. The four fluorescence acceptors, in the dNTP-attached forms, should each have a unique fluorescence emission distinguishable from that of the other three. Several sites on dNTPs will be explored for the attachment of the fluorescence acceptors. The initial efforts will be directed to the tagging of the terminal phosphate of dNTP. This approach has a unique advantage. When the incoming, tagged dNTP is bound to the active site of the polymerase, significant FRET from the donor on the polymerase to the acceptor on the dNTP is expected to occur. The unique fluorescence of the acceptor then enables the determination of the identity of the dNTP. Once the tagged dNTP is processed for covalent attachment to the nascent DNA chain, the fluorescence acceptor remains attached to the pyrophosphate and will be released to the medium. In fact, the growing nascent DNA chain will contain only the normal dNMP building units and no fluorescence acceptor molecules at all. In essence, FRET will only occur between the donor on the polymerase and incoming acceptor-labeled dNTP, one at a time. This approach is better than the alternative attachment of the acceptor to any site within the dNMP moiety of the initial dNTPs. In this latter case, the nascent DNA chain will contain multiple molecules of the fluorescence acceptors. Interference with the polymerase reaction and FRET measurements could occur. **P17,¶3&4** **P15,*Prediction of FRET efficiency***
78.(**previously presented**) The composition of claim 73,		

	wherein the polymerase comprises *Taq* DNA polymerase I having a tag attached to an amino acid at a specific amino acid position of the *Taq* DNA polymerase I, where the amino acid position is selected from the group consisting of 513-518, 643, 647, 649 and 653-661 of SEQ. ID No. 11, where the tag comprises a fluorescent molecule.	***Note: Only in Non-provisional; however, provisional fully disclosed the mechanism by which these sites were selected.*** The identification of sites in the polymerase that are not in contact with other proteins, that should not alter the conformation or folding of the protein, and that are not involved in the function of the protein, will be accomplished by a combination of sequence analyses and molecular docking studies. Regions of the protein surface that are not important for function can be identified, indirectly, by investigating the variation in sequence as a function of evolutionary time and protein function, with use of the evolutionary trace method (Lichtarge et al., 1996). In this approach, amino acid residues that are important for structure or function are found by comparing evolutionary mutations and structural homologies. The polymerases are ideal systems for this type of study, as there are many crystal and co-crystal structures and many available sequences. We will exclude the regions of structural/functional importance from consideration as sites for mutation/labeling. In addition, visual inspection and overlays of available structures in different conformational states, as already available from crystallographic studies, will further assist in identifying areas near the binding site for dNTPs that might be available for mutation and labeling. We envision choosing amino acids somewhat internally located, perhaps surrounding the enzyme active site, to reduce background (i.e. enzyme interacting with non-specifically associated dNTPs). Mutated and labeled polymerases will be built and energy minimized in a full solvent environment to estimate the effect on the structure of the mutation and/or labeling. This will also provide an estimate of the orientation of the fluorescent label with respect to the dNTP-binding pocket, thereby allowing us to estimate the FRET efficiency prior to measurement. **P13,¶3**
79.(**currently amended**) A composition comprising		

	a polymerizing agent including a molecular tag covalently bonded to a site on the polymerizing agent	In this method a single tag that is strategically positioned on a DNA polymerase interacts with a color-coded dNTP. **P3,¶1** The chemical attachment of a fluorescence donor to the engineered polymerase and characterization of the modified enzymes will be carried out in Dr. Tu's lab. **P3,¶2** We envision placing a single tag on the polymerase and a unique tag on each dNTP. **P6,¶1** In the first approach, we envision placing a fluorescence donor on the polymerase (i.e. fluorescein or fluorescein-type molecule) and a fluorescence acceptor with a unique fluorescent tag color on each dNTP (i.e. d-rhodamine or similar molecule). **P10,¶1** In one approach, a fluorescence donor will be attached to the polymerase and four unique fluorescence acceptors will each be attached to a different dNTP. The absorption spectra of the donor and acceptor fluorophores should be sufficiently distinct to allow exclusive (preferably) or preferential excitation of the fluorescence donor attached on the polymerase at a chosen wavelength. The emission of the fluorescence donor should have significant overlap with the absorption spectra of the fluorescence acceptors. The four fluorescence acceptors, in the dNTP-attached forms, should each have a unique fluorescence emission distinguishable from that of the other three. **P17,Approach I**
	lacking 3' to 5' exonuclease activity and	Taq DNA polymerase lacks a 3' to 5' exonuclease activity (proofreading activity). This is important for our studies since we do not want the enzyme to remove a base for which fluorescent signal was detected. If the enzyme used in single-molecule DNA sequencing possessed a 3' to 5' exonuclease activity, the enzyme would add another base to replace of the one that had been removed. This newly added base would produce a signature fluorescent signal that would suggest the presence of two identical bases in the template. This type of artifact could be detrimental to the technology. **P12,¶3**

	a monomer including a molecular tag that is released upon monomer incorporation,	In this method a single tag that is strategically positioned on a DNA polymerase interacts with a color-coded dNTP. **P3,¶1** Once the optimized enzyme is identified, it will be used to stimulate fluorescence transfer with an incoming dNTP (design of detection equipment, Dr. R. Willson; choices of fluorescent donors and acceptors, Drs. D. Tu and X. Gao; choice of site for labeling dNTP, Drs. Briggs, Hardin, Tu, Gao). **P3,¶2** Dr. Gao's group will design, synthesize, and purify tagged dNTPs (base, sugar, or phosphate labeled). **P4,¶4** We envision placing a single tag on the polymerase and a unique tag on each dNTP. **P6,¶1** In the first approach, we envision placing a fluorescence donor on the polymerase (i.e. fluorescein or fluorescein-type molecule) and a fluorescence acceptor with a unique fluorescent tag color on each dNTP (i.e. d-rhodamine or similar molecule). **P10,¶1** One of the ideas presented in this proposal is to put the fluorescent tag on the γ-phosphate such that, upon base incorporation, the tagged PPi will diffuse away from the protein (i.e. FRET will cease). According to our preliminary modeling studies, and the GTP/protein complex model presented in Figure 4 of Kiefer et al., 1998, there appears to be sufficient room for a tag on the γ-phosphate, without inhibiting incorporation. **P14-15,Last ¶** In one approach, a fluorescence donor will be attached to the polymerase and four unique fluorescence acceptors will each be attached to a different dNTP. The absorption spectra of the donor and acceptor fluorophores should be sufficiently distinct to allow exclusive (preferably) or preferential excitation of the fluorescence donor attached on the polymerase at a chosen wavelength. The emission of the fluorescence donor should have significant overlap with the absorption spectra of the fluorescence acceptors. The four fluorescence acceptors, in the dNTP-attached forms, should each have a unique fluorescence emission distinguishable from that of the other three. **P17,Approach I** **P20, *Scheme 3***

	where at least one of the tags has a fluorescence property that undergoes a change before, during and/or after each of a sequence of monomer incorporations due to an interaction between the polymerizing agent tag and the monomer tag and	As the correct dNTP is incorporated during the polymerization reaction, the identity of the base is indicated by a signature fluorescent signal. **P3,¶1** Once the optimized enzyme is identified, it will be used to stimulate fluorescence transfer with an incoming dNTP (design of detection equipment, Dr. R. Willson; choices of fluorescent donors and acceptors, Drs. D. Tu and X. Gao; choice of site for labeling dNTP, Drs. Briggs, Hardin, Tu, Gao). **P3,¶2** Dr. Briggs' effort includes identification of candidate amino acids for targeted mutagenesis of the polymerase via modeling of the complex between the (labeled) dNTP and the (labeled) protein. The efficiency of the fluorescence resonance energy transfer (FRET) will be predicted. Dr. Briggs' group will also work closely with Dr. Hardin's group to create the base identification software. **P4,¶5** We envision placing a single tag on the polymerase and a unique tag on each dNTP. **P6,¶1** As incoming fluorescently-tagged dNTP is bound by the polymerase for DNA elongation, a characteristic fluorescent signal is emitted that indicates base identity (emission wavelength and intensity provide signature for base identity). **P10,¶1** Mutated and labeled polymerases will be built and energy minimized in a full solvent environment to estimate the effect on the structure of the mutation and/or labeling. This will also provide an estimate of the orientation of the fluorescent label with respect to the dNTP-binding pocket, thereby allowing us to estimate the FRET efficiency prior to measurement. **P13,¶3** In one approach, a fluorescence donor will be attached to the polymerase and four unique fluorescence acceptors will each be attached to a different dNTP. The absorption spectra of the donor and acceptor fluorophores should be sufficiently distinct to allow exclusive (preferably) or preferential excitation of the fluorescence donor attached on the polymerase at a chosen wavelength. The emission of the fluorescence donor should have significant overlap with the absorption spectra of the fluorescence acceptors. The four fluorescence acceptors, in the dNTP-attached forms, should each have a unique fluorescence emission distinguishable from that of the other three. **P17,Approach I**

	where the site comprises a naturally occurring cysteine site or a cysteine replacement site in the polymerizing agent selected so that the site is less than or equal to about 50Å from a tag on each incorporating monomer and is a site that is not involved in the function of the polymerizing agent and	We will monitor the activity of polymerase variants throughout enzyme development. Enzyme activity will be assayed after a candidate amino acid is mutated to cysteine and following fluorescent tagging of that cysteine. A similar assay will be used to monitor the ability of a polymerase or a polymerase variant to incorporate fluorescently-tagged dNTPs. Since the enzyme's amino acid sequence will be altered, we will determine whether enzyme characteristics are altered (thermostability, fidelity, polymerization rate, affinity for modified versus natural bases). Similar procedures will be used to identify the optimal reaction buffer. **P16,¶5** **P15,*Prediction of FRET efficiency***

	the polymerizing agent tag is covalently bonded to the naturally occurring cysteine site or the cysteine replacement site through its SH group.	A key task for this project is the identification of amino acids in the polymerase that can withstand mutation and fluorescent labeling. This will be accomplished via a combination of computational methods, mutational studies, and assaying for normal protein function. Follow-up computational analyses will be performed to refine the molecular models such that they might be used to help suggest alternative sites for incorporation of a fluorescent tag in the event that problems are encountered with the preliminary suggestions. The identification of sites in the polymerase that are not in contact with other proteins, that should not alter the conformation or folding of the protein, and that are not involved in the function of the protein, will be accomplished by a combination of sequence analyses and molecular docking studies. Regions of the protein surface that are not important for function can be identified, indirectly, by investigating the variation in sequence as a function of evolutionary time and protein function, with use of the evolutionary trace method (Lichtarge et al., 1996). In this approach, amino acid residues that are important for structure or function are found by comparing evolutionary mutations and structural homologies. The polymerases are ideal systems for this type of study, as there are many crystal and co-crystal structures and many available sequences. We will exclude the regions of structural/functional importance from consideration as sites for mutation/labeling. In addition, visual inspection and overlays of available structures in different conformational states, as already available from crystallographic studies, will further assist in identifying areas near the binding site for dNTPs that might be available for mutation and labeling. We envision choosing amino acids somewhat internally located, perhaps surrounding the enzyme active site, to reduce background (i.e. enzyme interacting with non-specifically associated dNTPs). Mutated and labeled polymerases will be built and energy minimized in a full solvent environment to estimate the effect on the structure of the mutation and/or labeling. This will also provide an estimate of the orientation of the fluorescent label with respect to the dNTP-binding pocket, thereby allowing us to estimate the FRET efficiency prior to measurement. **P13,2&3**

		We will monitor the activity of polymerase variants throughout enzyme development. Enzyme activity will be assayed after a candidate amino acid is mutated to cysteine and following fluorescent tagging of that cysteine. A similar assay will be used to monitor the ability of a polymerase or a polymerase variant to incorporate fluorescently-tagged dNTPs. Since the enzyme's amino acid sequence will be altered, we will determine whether enzyme characteristics are altered (thermostability, fidelity, polymerization rate, affinity for modified versus natural bases). Similar procedures will be used to identify the optimal reaction buffer. **P16,¶5**

		In general, the polymerase with specific residue(s) targeted or created mutationally for labeling will be treated with a slight molar excess of a desired probe in the hope that near stoichiometric labeling can be achieved. Alternatively, the polymerase can be treated with an excess amount of the probe and the labeling will be followed as a function of time. The tagging reaction will be stopped when near stoichiometric labeling is obtained. The possibility that excessive tagging of residues other than the targeted one occurs leading to adverse effects on enzyme activity or subsequent FRET measurement should be considered. If the targeted residue is close to the active site, a saturating level of substrate or a competitive inhibitor can first be added to protect the targeted residue at the enzyme active site and a reversible labeling reagent can be subsequently added to tag these non-active site residues. The modified enzyme will be freed from the protective substrate (or competitive inhibitor) and remaining free reversible reagent, and treated with the desired fluorescence probe for the labeling of the targeted residue. Finally, the reversible tags will be chemically freed from the enzyme and removed to obtain the polymerase containing the desired fluorescence donor attached to primarily the targeted residue. Alternatively, the targeted residue may not be near the active site. Excessive labeling of other residues could only occur if they are significantly more reactive than the targeted residue for tagging. The polymerase can also be treated with a reversible reagent for preferential labeling of those residues which are not selected for fluorescence probe attachment, but are chemically more susceptible for tagging. After removal of the remaining free reversible reagent, the modified enzyme can then be treated with the desired fluorescence probe for the labeling of the less reactive targeted residue. Finally, the reversible tags can be chemically freed from the enzyme and removed to obtain the polymerase with the fluorescence probe attached to primarily the targeted residue. **P18, ¶2**
80.(**previously presented**) The composition of claim 79,		
	wherein the site is less than or equal to about 15Å from a tag on each incorporating monomer.	**Not specifically in the proposal. But the placement of the donor on the polymerase relative to the acceptor on incorporating monomer is not disclosed by either Korlach et al. or Schneider et al.**

81.(**previously presented**) The composition of claim 79,	
wherein the site is less than or equal to about 10Å from a tag on each incorporating monomer.	**Not specifically in the proposal. But the placement of the donor on the polymerase relative to the acceptor on incorporating monomer is not disclosed by either Korlach et al. or Schneider et al.**
82.(**previously presented**) The composition of claim 79,	
wherein the polymerizing agent is a polymerase or reverse transcriptase.	***Note polymerases are disclosed throughout proposal*** In this method a single tag that is strategically positioned on a DNA polymerase interacts with a color-coded dNTP. **P3,¶1** The chemical attachment of a fluorescence donor to the engineered polymerase and characterization of the modified enzymes will be carried out in Dr. Tu's lab. P3,¶2 We envision placing a single tag on the polymerase and a unique tag on each dNTP. **P6,¶1** **P10,Enzyme Choice through paragraph bridging P14&15** ***Note: reverse transcriptases were not disclosed,but are a type of polymerase***
83.(**previously presented**) The composition of claim 79,	

	wherein the polymerase is selected from the group consisting of *Taq* DNA polymerase I, T7 DNA polymerase, Sequenase, and the Klenow fragment from *E. coli* DNA polymerase I.	Although crystal structures are available and the enzyme does not contain naturally occurring cysteines, native Taq DNA polymerase is not optimally suited for our purposes since it is not a very processive polymerase (50-80 nucleotides are incorporated before dissociation). It can, however, be appropriately engineered. Specifically, development of a single-molecule DNA sequencer will benefit by using a DNA polymerase that remains associated with the DNA template during the extension phase of the sequencing reaction. Using a highly processive enzyme is expected to minimize complications that may arise from dissociation from the template, which will alter the polymerization rate. However, these rate differences could be compensated for by appropriately modifying the base calling software. Thus, lack of processivity may not limit the sequence lengths achievable by this invention. This feature - processivity - of the native Taq enzyme could negatively impact sequencing run lengths. However, enzymes responsible for replicating the genome are very processive and are able to replicate thousands of bases before dissociating from the template (Kornberg and Baker, 1992). In fact, eukaryotic and prokaryotic DNA polymerases possess mechanisms to overcoming this shortcoming: Increased processivity is achieved through the use of accessory factors (Kelman et al., 1998). A particularly relevant example involves T7 DNA polymerase and its interaction with thioredoxin, a 12 kDa protein produced by E. coli. These proteins associate to form a complex that effectively encircles the DNA template, anchoring the replication complex to the template and achieving a several thousand-fold increase in processivity of T7 DNA polymerase (Tabor et al., 1987; Huber et al., 1987). Processivity can also be altered through genetic engineering, as was elegantly demonstrated using the Klenow fragment from E. coli DNA polymerase I, a polymerase with even lower processivity than Taq. Increased processivity was obtained by introducing the 76 amino acid `processivity domain' from T7 DNA polymerase into the Klenow fragment (Bedford et al., 1997; Bedford et al., 1999). More specifically, this processivity domain contains the thioredoxin binding domain (TBD) from T7 DNA polymerase and it was engineered into the Klenow fragment between the H and H, helices (at the tip of 'thumb' region within the polymerase). This sequence addition caused a thioredoxin-dependent increase in both the processivity and specific activity of Klenow fragment. Thus, we propose to introduce this same region of T7 DNA polymerase into the homologous site of Taq DNA polymerase (Bedford et al., 1999). If necessary, the TBD and thioredoxin can be altered to become more heat stable. **P11,¶3-5**

84.(**currently amended**) The composition of claim ~~83~~82,	
wherein the reverse transcriptase comprises HIV-1 reverse transcriptase.	***Not disclosed in the proposal, but this enzyme is a type of polymerase.***
85.(**currently amended**) The composition of claim 79,	

	wherein each of the monomers comprises a deoxynucleotide triphosphate (dNTP) and the monomer tag is covalently bonded directly or through a linker to the ~~β and/or γ phosphate group~~ pyrophosphate moiety of each dNTP.	In this method a single tag that is strategically positioned on a DNA polymerase interacts with a color-coded dNTP. **P3,¶1** Once the optimized enzyme is identified, it will be used to stimulate fluorescence transfer with an incoming dNTP (design of detection equipment, Dr. R. Willson; choices of fluorescent donors and acceptors, Drs. D. Tu and X. Gao; choice of site for labeling dNTP, Drs. Briggs, Hardin, Tu, Gao). **P3,¶2** Dr. Gao's group will design, synthesize, and purify tagged dNTPs (base, sugar, or phosphate labeled). **P4,¶4** We envision placing a single tag on the polymerase and a unique tag on each dNTP. **P6,¶1** In the first approach, we envision placing a fluorescence donor on the polymerase (i.e. fluorescein or fluorescein-type molecule) and a fluorescence acceptor with a unique fluorescent tag color on each dNTP (i.e. d-rhodamine or similar molecule). **P10,¶1** One of the ideas presented in this proposal is to put the fluorescent tag on the γ-phosphate such that, upon base incorporation, the tagged PPi will diffuse away from the protein (i.e. FRET will cease). According to our preliminary modeling studies, and the GTP/protein complex model presented in Figure 4 of Kiefer et al., 1998, there appears to be sufficient room for a tag on the γ-phosphate, without inhibiting incorporation. **P14-15,Last ¶** In one approach, a fluorescence donor will be attached to the polymerase and four unique fluorescence acceptors will each be attached to a different dNTP. The absorption spectra of the donor and acceptor fluorophores should be sufficiently distinct to allow exclusive (preferably) or preferential excitation of the fluorescence donor attached on the polymerase at a chosen wavelength. The emission of the fluorescence donor should have significant overlap with the absorption spectra of the fluorescence acceptors. The four fluorescence acceptors, in the dNTP-attached forms, should each have a unique fluorescence emission distinguishable from that of the other three. **P17,Approach I** **P20, *Scheme 3***
86.(**currently amended**)	The composition of claim 85,	

	wherein the tags comprise fluorescent tags and the fluorescence property comprises a duration, an intensity and/or frequency of emitted fluorescent light.	As the correct dNTP is incorporated during the polymerization reaction, the identity of the base is indicated by a signature fluorescent signal. **P3,¶1** As a tagged dNTP is incorporated into the DNA polymer, a characteristic fluorescent signal is emitted that indicates base identity (emission wavelength and/or strength provide signature for base identity). . . . The fluorescent signals produced upon incorporation will be detected and analyzed to determine DNA base sequence. **P6,¶1** As incoming fluorescently-tagged dNTP is bound by the polymerase for DNA elongation, a characteristic fluorescent signal is emitted that indicates base identity (emission wavelength and intensity provide signature for base identity). **P10,¶1** The raw data generated by the detector will represent between one to four time-dependent data streams of fluorescence wavelengths and intensities, one data stream for each fluorescently labeled base (i.e. wavelength) being monitored. **P22,¶1**
87.(**previously presented**) The composition of claim 86,		

	wherein the fluorescence property is fluorescence resonance energy transfer (FRET) where either the monomer tag or the polymerase tag comprises a donor and the other tag comprises an acceptor and where FRET occurs when the two tags are in close proximity.	***Approach I*** The following principles will be guiding our search for appropriate fluorescence dyes for this work. In one approach, a fluorescence donor will be attached to the polymerase and four unique fluorescence acceptors will each be attached to a different dNTP. The absorption spectra of the donor and acceptor fluorophores should be sufficiently distinct to allow exclusive (preferably) or preferential excitation of the fluorescence donor attached on the polymerase at a chosen wavelength. The emission of the fluorescence donor should have significant overlap with the absorption spectra of the fluorescence acceptors. The four fluorescence acceptors, in the dNTP-attached forms, should each have a unique fluorescence emission distinguishable from that of the other three. Several sites on dNTPs will be explored for the attachment of the fluorescence acceptors. The initial efforts will be directed to the tagging of the terminal phosphate of dNTP. This approach has a unique advantage. When the incoming, tagged dNTP is bound to the active site of the polymerase, significant FRET from the donor on the polymerase to the acceptor on the dNTP is expected to occur. The unique fluorescence of the acceptor then enables the determination of the identity of the dNTP. Once the tagged dNTP is processed for covalent attachment to the nascent DNA chain, the fluorescence acceptor remains attached to the pyrophosphate and will be released to the medium. In fact, the growing nascent DNA chain will contain only the normal dNMP building units and no fluorescence acceptor molecules at all. In essence, FRET will only occur between the donor on the polymerase and incoming acceptor-labeled dNTP, one at a time. This approach is better than the alternative attachment of the acceptor to any site within the dNMP moiety of the initial dNTPs. In this latter case, the nascent DNA chain will contain multiple molecules of the fluorescence acceptors. Interference with the polymerase reaction and FRET measurements could occur. **P17,¶3&4** **P15,*Prediction of FRET efficiency***
88.(**previously presented**) The composition of claim 83,		

	wherein the polymerase comprises *Taq* DNA polymerase I having a tag attached to an amino acid at a specific amino acid position of the *Taq* DNA polymerase I, where the amino acid position is selected from the group consisting of 513-518, 643, 647, 649 and 653-661 of SEQ. ID No. 11, where the tag comprises a fluorescent molecule.	***Note: Only in Non-provisional; however, provisional fully disclosed the mechanism by which these sites were selected.*** The identification of sites in the polymerase that are not in contact with other proteins, that should not alter the conformation or folding of the protein, and that are not involved in the function of the protein, will be accomplished by a combination of sequence analyses and molecular docking studies. Regions of the protein surface that are not important for function can be identified, indirectly, by investigating the variation in sequence as a function of evolutionary time and protein function, with use of the evolutionary trace method (Lichtarge et al., 1996). In this approach, amino acid residues that are important for structure or function are found by comparing evolutionary mutations and structural homologies. The polymerases are ideal systems for this type of study, as there are many crystal and co-crystal structures and many available sequences. We will exclude the regions of structural/functional importance from consideration as sites for mutation/labeling. In addition, visual inspection and overlays of available structures in different conformational states, as already available from crystallographic studies, will further assist in identifying areas near the binding site for dNTPs that might be available for mutation and labeling. We envision choosing amino acids somewhat internally located, perhaps surrounding the enzyme active site, to reduce background (i.e. enzyme interacting with non-specifically associated dNTPs). Mutated and labeled polymerases will be built and energy minimized in a full solvent environment to estimate the effect on the structure of the mutation and/or labeling. This will also provide an estimate of the orientation of the fluorescent label with respect to the dNTP-binding pocket, thereby allowing us to estimate the FRET efficiency prior to measurement. **P13,¶3**
89.(**currently amended**) A composition comprising a		

	polymerizing agent including a molecular tag covalently bonded to a site on the polymerizing agent and	In this method a single tag that is strategically positioned on a DNA polymerase interacts with a color-coded dNTP. **P3,¶1** The chemical attachment of a fluorescence donor to the engineered polymerase and characterization of the modified enzymes will be carried out in Dr. Tu's lab. **P3,¶2** We envision placing a single tag on the polymerase and a unique tag on each dNTP. **P6,¶1** In the first approach, we envision placing a fluorescence donor on the polymerase (i.e. fluorescein or fluorescein-type molecule) and a fluorescence acceptor with a unique fluorescent tag color on each dNTP (i.e. d-rhodamine or similar molecule). **P10,¶1** In one approach, a fluorescence donor will be attached to the polymerase and four unique fluorescence acceptors will each be attached to a different dNTP. The absorption spectra of the donor and acceptor fluorophores should be sufficiently distinct to allow exclusive (preferably) or preferential excitation of the fluorescence donor attached on the polymerase at a chosen wavelength. The emission of the fluorescence donor should have significant overlap with the absorption spectra of the fluorescence acceptors. The four fluorescence acceptors, in the dNTP-attached forms, should each have a unique fluorescence emission distinguishable from that of the other three. **P17,Approach I**

	a monomer including a molecular tag covalently bonded to the monomer and that is released upon monomer incorporation,	In this method a single tag that is strategically positioned on a DNA polymerase interacts with a color-coded dNTP. **P3,¶1** Once the optimized enzyme is identified, it will be used to stimulate fluorescence transfer with an incoming dNTP (design of detection equipment, Dr. R. Willson; choices of fluorescent donors and acceptors, Drs. D. Tu and X. Gao; choice of site for labeling dNTP, Drs. Briggs, Hardin, Tu, Gao). **P3,¶2** Dr. Gao's group will design, synthesize, and purify tagged dNTPs (base, sugar, or phosphate labeled). **P4,¶4** We envision placing a single tag on the polymerase and a unique tag on each dNTP. **P6,¶1** In the first approach, we envision placing a fluorescence donor on the polymerase (i.e. fluorescein or fluorescein-type molecule) and a fluorescence acceptor with a unique fluorescent tag color on each dNTP (i.e. d-rhodamine or similar molecule). **P10,¶1** One of the ideas presented in this proposal is to put the fluorescent tag on the γ-phosphate such that, upon base incorporation, the tagged PPi will diffuse away from the protein (i.e. FRET will cease). According to our preliminary modeling studies, and the GTP/protein complex model presented in Figure 4 of Kiefer et al., 1998, there appears to be sufficient room for a tag on the γ-phosphate, without inhibiting incorporation. **P14-15,Last ¶** In one approach, a fluorescence donor will be attached to the polymerase and four unique fluorescence acceptors will each be attached to a different dNTP. The absorption spectra of the donor and acceptor fluorophores should be sufficiently distinct to allow exclusive (preferably) or preferential excitation of the fluorescence donor attached on the polymerase at a chosen wavelength. The emission of the fluorescence donor should have significant overlap with the absorption spectra of the fluorescence acceptors. The four fluorescence acceptors, in the dNTP-attached forms, should each have a unique fluorescence emission distinguishable from that of the other three. **P17,Approach I** **P20, *Scheme 3***

	where at least one of the tags has a fluorescence property that undergoes a change before, during and/or after each of a sequence of monomer incorporations due to an interaction between the polymerizing agent tag and the monomer tag and	As the correct dNTP is incorporated during the polymerization reaction, the identity of the base is indicated by a signature fluorescent signal. **P3,¶1** Once the optimized enzyme is identified, it will be used to stimulate fluorescence transfer with an incoming dNTP (design of detection equipment, Dr. R. Willson; choices of fluorescent donors and acceptors, Drs. D. Tu and X. Gao; choice of site for labeling dNTP, Drs. Briggs, Hardin, Tu, Gao). **P3,¶2** Dr. Briggs' effort includes identification of candidate amino acids for targeted mutagenesis of the polymerase via modeling of the complex between the (labeled) dNTP and the (labeled) protein. The efficiency of the fluorescence resonance energy transfer (FRET) will be predicted. Dr. Briggs' group will also work closely with Dr. Hardin's group to create the base identification software. **P4,¶5** We envision placing a single tag on the polymerase and a unique tag on each dNTP. **P6,¶1** As incoming fluorescently-tagged dNTP is bound by the polymerase for DNA elongation, a characteristic fluorescent signal is emitted that indicates base identity (emission wavelength and intensity provide signature for base identity). P10,¶1 Mutated and labeled polymerases will be built and energy minimized in a full solvent environment to estimate the effect on the structure of the mutation and/or labeling. This will also provide an estimate of the orientation of the fluorescent label with respect to the dNTP-binding pocket, thereby allowing us to estimate the FRET efficiency prior to measurement. **P13,¶3** In one approach, a fluorescence donor will be attached to the polymerase and four unique fluorescence acceptors will each be attached to a different dNTP. The absorption spectra of the donor and acceptor fluorophores should be sufficiently distinct to allow exclusive (preferably) or preferential excitation of the fluorescence donor attached on the polymerase at a chosen wavelength. The emission of the fluorescence donor should have significant overlap with the absorption spectra of the fluorescence acceptors. The four fluorescence acceptors, in the dNTP-attached forms, should each have a unique fluorescence emission distinguishable from that of the other three. **P17,Approach I**

	where the site comprises a naturally occurring cysteine site or a cysteine replacement site in the polymerizing agent selected so that the site is less than or equal to about 50Å from a tag on each incorporating	We will monitor the activity of polymerase variants throughout enzyme development. Enzyme activity will be assayed after a candidate amino acid is mutated to cysteine and following fluorescent tagging of that cysteine. A similar assay will be used to monitor the ability of a polymerase or a polymerase variant to incorporate fluorescently-tagged dNTPs. Since the enzyme's amino acid sequence will be altered, we will determine whether enzyme characteristics are altered (thermostability, fidelity, polymerization rate, affinity for modified versus natural bases). Similar procedures will be used to identify the optimal reaction buffer. **P16,¶5** **P15,*Prediction of FRET efficiency***

	monomer and the polymerizing agent tag is covalently bonded to the naturally occurring cysteine site or the cysteine replacement site through its SH group.	A key task for this project is the identification of amino acids in the polymerase that can withstand mutation and fluorescent labeling. This will be accomplished via a combination of computational methods, mutational studies, and assaying for normal protein function. Follow-up computational analyses will be performed to refine the molecular models such that they might be used to help suggest alternative sites for incorporation of a fluorescent tag in the event that problems are encountered with the preliminary suggestions. The identification of sites in the polymerase that are not in contact with other proteins, that should not alter the conformation or folding of the protein, and that are not involved in the function of the protein, will be accomplished by a combination of sequence analyses and molecular docking studies. Regions of the protein surface that are not important for function can be identified, indirectly, by investigating the variation in sequence as a function of evolutionary time and protein function, with use of the evolutionary trace method (Lichtarge et al., 1996). In this approach, amino acid residues that are important for structure or function are found by comparing evolutionary mutations and structural homologies. The polymerases are ideal systems for this type of study, as there are many crystal and co-crystal structures and many available sequences. We will exclude the regions of structural/functional importance from consideration as sites for mutation/labeling. In addition, visual inspection and overlays of available structures in different conformational states, as already available from crystallographic studies, will further assist in identifying areas near the binding site for dNTPs that might be available for mutation and labeling. We envision choosing amino acids somewhat internally located, perhaps surrounding the enzyme active site, to reduce background (i.e. enzyme interacting with non-specifically associated dNTPs). Mutated and labeled polymerases will be built and energy minimized in a full solvent environment to estimate the effect on the structure of the mutation and/or labeling. This will also provide an estimate of the orientation of the fluorescent label with respect to the dNTP-binding pocket, thereby allowing us to estimate the FRET efficiency prior to measurement. **P13,2&3**

		We will monitor the activity of polymerase variants throughout enzyme development. Enzyme activity will be assayed after a candidate amino acid is mutated to cysteine and following fluorescent tagging of that cysteine. A similar assay will be used to monitor the ability of a polymerase or a polymerase variant to incorporate fluorescently-tagged dNTPs. Since the enzyme's amino acid sequence will be altered, we will determine whether enzyme characteristics are altered (thermostability, fidelity, polymerization rate, affinity for modified versus natural bases). Similar procedures will be used to identify the optimal reaction buffer. **P16,¶5**

		In general, the polymerase with specific residue(s) targeted or created mutationally for labeling will be treated with a slight molar excess of a desired probe in the hope that near stoichiometric labeling can be achieved. Alternatively, the polymerase can be treated with an excess amount of the probe and the labeling will be followed as a function of time. The tagging reaction will be stopped when near stoichiometric labeling is obtained. The possibility that excessive tagging of residues other than the targeted one occurs leading to adverse effects on enzyme activity or subsequent FRET measurement should be considered. If the targeted residue is close to the active site, a saturating level of substrate or a competitive inhibitor can first be added to protect the targeted residue at the enzyme active site and a reversible labeling reagent can be subsequently added to tag these non-active site residues. The modified enzyme will be freed from the protective substrate (or competitive inhibitor) and remaining free reversible reagent, and treated with the desired fluorescence probe for the labeling of the targeted residue. Finally, the reversible tags will be chemically freed from the enzyme and removed to obtain the polymerase containing the desired fluorescence donor attached to primarily the targeted residue. Alternatively, the targeted residue may not be near the active site. Excessive labeling of other residues could only occur if they are significantly more reactive than the targeted residue for tagging. The polymerase can also be treated with a reversible reagent for preferential labeling of those residues which are not selected for fluorescence probe attachment, but are chemically more susceptible for tagging. After removal of the remaining free reversible reagent, the modified enzyme can then be treated with the desired fluorescence probe for the labeling of the less reactive targeted residue. Finally, the reversible tags can be chemically freed from the enzyme and removed to obtain the polymerase with the fluorescence probe attached to primarily the targeted residue. **P18, ¶2**
90.(**previously presented**) The composition of claim 89,		
	wherein the site is less than or equal to about 15Å from a tag on each incorporating monomer.	**Not specifically in the proposal. But the placement of the donor on the polymerase relative to the acceptor on incorporating monomer is not disclosed by either Korlach et al. or Schneider et al.**

91.(**previously presented**) The composition of claim 89,	
wherein the site is less than or equal to about 10Å from a tag on each incorporating monomer.	**Not specifically in the proposal. But the placement of the donor on the polymerase relative to the acceptor on incorporating monomer is not disclosed by either Korlach et al. or Schneider et al.**
92.(**previously presented**) The composition of claim 89,	
wherein the polymerizing agent is a polymerase or reverse transcriptase.	***Note polymerases are disclosed throughout proposal*** In this method a single tag that is strategically positioned on a DNA polymerase interacts with a color-coded dNTP. **P3,¶1** The chemical attachment of a fluorescence donor to the engineered polymerase and characterization of the modified enzymes will be carried out in Dr. Tu's lab. **P3,¶2** We envision placing a single tag on the polymerase and a unique tag on each dNTP. **P6,¶1** **P10,Enzyme Choice through paragraph bridging P14&15** ***Note: reverse transcriptases were not disclosed, but are a type of polymerase***
94.(**previously presented**) The composition of claim 92,	

	wherein the polymerase is selected from the group consisting of *Taq* DNA polymerase I, T7 DNA polymerase, Sequenase, and the Klenow fragment from *E. coli* DNA polymerase I.	Although crystal structures are available and the enzyme does not contain naturally occurring cysteines, native Taq DNA polymerase is not optimally suited for our purposes since it is not a very processive polymerase (50-80 nucleotides are incorporated before dissociation). It can, however, be appropriately engineered. Specifically, development of a single-molecule DNA sequencer will benefit by using a DNA polymerase that remains associated with the DNA template during the extension phase of the sequencing reaction. Using a highly processive enzyme is expected to minimize complications that may arise from dissociation from the template, which will alter the polymerization rate. However, these rate differences could be compensated for by appropriately modifying the base calling software. Thus, lack of processivity may not limit the sequence lengths achievable by this invention. This feature - processivity - of the native Taq enzyme could negatively impact sequencing run lengths. However, enzymes responsible for replicating the genome are very processive and are able to replicate thousands of bases before dissociating from the template (Kornberg and Baker, 1992). In fact, eukaryotic and prokaryotic DNA polymerases possess mechanisms to overcoming this shortcoming: Increased processivity is achieved through the use of accessory factors (Kelman et al., 1998). A particularly relevant example involves T7 DNA polymerase and its interaction with thioredoxin, a 12 kDa protein produced by E. coli. These proteins associate to form a complex that effectively encircles the DNA template, anchoring the replication complex to the template and achieving a several thousand-fold increase in processivity of T7 DNA polymerase (Tabor et al., 1987; Huber et al., 1987). Processivity can also be altered through genetic engineering, as was elegantly demonstrated using the Klenow fragment from E. coli DNA polymerase I, a polymerase with even lower processivity than Taq. Increased processivity was obtained by introducing the 76 amino acid `processivity domain' from T7 DNA polymerase into the Klenow fragment (Bedford et al., 1997; Bedford et al., 1999). More specifically, this processivity domain contains the thioredoxin binding domain (TBD) from T7 DNA polymerase and it was engineered into the Klenow fragment between the H and H, helices (at the tip of 'thumb' region within the polymerase). This sequence addition caused a thioredoxin-dependent increase in both the processivity and specific activity of Klenow fragment. Thus, we propose to introduce this same region of T7 DNA polymerase into the homologous site of Taq DNA polymerase (Bedford et al., 1999). If necessary, the TBD and thioredoxin can be altered to become more heat stable. **P11,¶3-5**

		Taq DNA polymerase lacks a 3' to 5' exonuclease activity (proofreading activity). This is important for our studies since we do not want the enzyme to remove a base for which fluorescent signal was detected. If the enzyme used in single-molecule DNA sequencing possessed a 3' to 5' exonuclease activity, the enzyme would add another base to replace of the one that had been removed. This newly added base would produce a signature fluorescent signal that would suggest the presence of two identical bases in the template. This type of artifact could be detrimental to the technology. **P12,¶3**
95.(**previously presented**) The composition of claim 92,		
	wherein the reverse transcriptase comprises HIV-1 reverse transcriptase.	***Not disclosed in the proposal, but this enzyme is a type of polymerase.***
96.(**previously presented**) The composition of claim 89,		

	wherein each of the monomers comprises a deoxynucleotide triphosphate (dNTP) and the monomer tag is covalently bonded directly or through a linker to the terminal phosphate group of each dNTP.	In this method a single tag that is strategically positioned on a DNA polymerase interacts with a color-coded dNTP. **P3,¶1** Once the optimized enzyme is identified, it will be used to stimulate fluorescence transfer with an incoming dNTP (design of detection equipment, Dr. R. Willson; choices of fluorescent donors and acceptors, Drs. D. Tu and X. Gao; choice of site for labeling dNTP, Drs. Briggs, Hardin, Tu, Gao). **P3,¶2** Dr. Gao's group will design, synthesize, and purify tagged dNTPs (base, sugar, or phosphate labeled). **P4,¶4** We envision placing a single tag on the polymerase and a unique tag on each dNTP. P6,¶1 In the first approach, we envision placing a fluorescence donor on the polymerase (i.e. fluorescein or fluorescein-type molecule) and a fluorescence acceptor with a unique fluorescent tag color on each dNTP (i.e. d-rhodamine or similar molecule). **P10,¶1** One of the ideas presented in this proposal is to put the fluorescent tag on the γ-phosphate such that, upon base incorporation, the tagged PPi will diffuse away from the protein (i.e. FRET will cease). According to our preliminary modeling studies, and the GTP/protein complex model presented in Figure 4 of Kiefer et al., 1998, there appears to be sufficient room for a tag on the γ-phosphate, without inhibiting incorporation. **P14-15,Last ¶** In one approach, a fluorescence donor will be attached to the polymerase and four unique fluorescence acceptors will each be attached to a different dNTP. The absorption spectra of the donor and acceptor fluorophores should be sufficiently distinct to allow exclusive (preferably) or preferential excitation of the fluorescence donor attached on the polymerase at a chosen wavelength. The emission of the fluorescence donor should have significant overlap with the absorption spectra of the fluorescence acceptors. The four fluorescence acceptors, in the dNTP-attached forms, should each have a unique fluorescence emission distinguishable from that of the other three. **P17,Approach I** **P20, *Scheme 3***
97.(**previously presented**) The composition of claim 96,		

	wherein the tags comprise fluorescent tags and the fluorescence property comprises a duration, an intensity and/or frequency of emitted fluorescent light.	As the correct dNTP is incorporated during the polymerization reaction, the identity of the base is indicated by a signature fluorescent signal. **P3,¶1** As a tagged dNTP is incorporated into the DNA polymer, a characteristic fluorescent signal is emitted that indicates base identity (emission wavelength and/or strength provide signature for base identity). . . . The fluorescent signals produced upon incorporation will be detected and analyzed to determine DNA base sequence. **P6,¶1** As incoming fluorescently-tagged dNTP is bound by the polymerase for DNA elongation, a characteristic fluorescent signal is emitted that indicates base identity (emission wavelength and intensity provide signature for base identity). **P10,¶1** The raw data generated by the detector will represent between one to four time-dependent data streams of fluorescence wavelengths and intensities, one data stream for each fluorescently labeled base (i.e. wavelength) being monitored. **P22,¶1**
98.(**previously presented**) The composition of claim 97,		

	wherein the fluorescence property is fluorescence resonance energy transfer (FRET) where either the monomer tag or the polymerase tag comprises a donor and the other tag comprises an acceptor and where FRET occurs when the two tags are in close proximity.	***Approach I*** The following principles will be guiding our search for appropriate fluorescence dyes for this work. In one approach, a fluorescence donor will be attached to the polymerase and four unique fluorescence acceptors will each be attached to a different dNTP. The absorption spectra of the donor and acceptor fluorophores should be sufficiently distinct to allow exclusive (preferably) or preferential excitation of the fluorescence donor attached on the polymerase at a chosen wavelength. The emission of the fluorescence donor should have significant overlap with the absorption spectra of the fluorescence acceptors. The four fluorescence acceptors, in the dNTP-attached forms, should each have a unique fluorescence emission distinguishable from that of the other three. Several sites on dNTPs will be explored for the attachment of the fluorescence acceptors. The initial efforts will be directed to the tagging of the terminal phosphate of dNTP. This approach has a unique advantage. When the incoming, tagged dNTP is bound to the active site of the polymerase, significant FRET from the donor on the polymerase to the acceptor on the dNTP is expected to occur. The unique fluorescence of the acceptor then enables the determination of the identity of the dNTP. Once the tagged dNTP is processed for covalent attachment to the nascent DNA chain, the fluorescence acceptor remains attached to the pyrophosphate and will be released to the medium. In fact, the growing nascent DNA chain will contain only the normal dNMP building units and no fluorescence acceptor molecules at all. In essence, FRET will only occur between the donor on the polymerase and incoming acceptor-labeled dNTP, one at a time. This approach is better than the alternative attachment of the acceptor to any site within the dNMP moiety of the initial dNTPs. In this latter case, the nascent DNA chain will contain multiple molecules of the fluorescence acceptors. Interference with the polymerase reaction and FRET measurements could occur. P17,¶3&4 P15,***Prediction of FRET efficiency***
99.(**previously presented**) The composition of claim 94,		

	wherein the polymerase comprises *Taq* DNA polymerase I having a tag attached to an amino acid at a specific amino acid position of the *Taq* DNA polymerase I, where the amino acid position is selected from the group consisting of 513-518, 643, 647, 649 and 653-661 of SEQ. ID No. 11, where the tag comprises a fluorescent molecule.	***Note: Only in Non-provisional; however, provisional fully disclosed the mechanism by which these sites were selected.*** The identification of sites in the polymerase that are not in contact with other proteins, that should not alter the conformation or folding of the protein, and that are not involved in the function of the protein, will be accomplished by a combination of sequence analyses and molecular docking studies. Regions of the protein surface that are not important for function can be identified, indirectly, by investigating the variation in sequence as a function of evolutionary time and protein function, with use of the evolutionary trace method (Lichtarge et al., 1996). In this approach, amino acid residues that are important for structure or function are found by comparing evolutionary mutations and structural homologies. The polymerases are ideal systems for this type of study, as there are many crystal and co-crystal structures and many available sequences. We will exclude the regions of structural/functional importance from consideration as sites for mutation/labeling. In addition, visual inspection and overlays of available structures in different conformational states, as already available from crystallographic studies, will further assist in identifying areas near the binding site for dNTPs that might be available for mutation and labeling. We envision choosing amino acids somewhat internally located, perhaps surrounding the enzyme active site, to reduce background (i.e. enzyme interacting with non-specifically associated dNTPs). Mutated and labeled polymerases will be built and energy minimized in a full solvent environment to estimate the effect on the structure of the mutation and/or labeling. This will also provide an estimate of the orientation of the fluorescent label with respect to the dNTP-binding pocket, thereby allowing us to estimate the FRET efficiency prior to measurement. **P13,¶3**
100.(**previously presented**) The composition of claim 50,		

	wherein the polymerizing agent lacks the ability to remove a previously incorporated monomer.	Taq DNA polymerase lacks a 3' to 5' exonuclease activity (proofreading activity). This is important for our studies since we do not want the enzyme to remove a base for which fluorescent signal was detected. If the enzyme used in single-molecule DNA sequencing possessed a 3' to 5' exonuclease activity, the enzyme would add another base to replace of the one that had been removed. This newly added base would produce a signature fluorescent signal that would suggest the presence of two identical bases in the template. This type of artifact could be detrimental to the technology. **P12,¶3**
102.(**previously presented**) The composition of claim 64,		
	wherein the polymerase lacks the ability to remove a previously incorporated monomer.	Taq DNA polymerase lacks a 3' to 5' exonuclease activity (proofreading activity). This is important for our studies since we do not want the enzyme to remove a base for which fluorescent signal was detected. If the enzyme used in single-molecule DNA sequencing possessed a 3' to 5' exonuclease activity, the enzyme would add another base to replace of the one that had been removed. This newly added base would produce a signature fluorescent signal that would suggest the presence of two identical bases in the template. This type of artifact could be detrimental to the technology. **P12,¶3**
103.(**previously presented**) The composition of claim 71,		
	wherein the polymerase lacks the ability to remove a previously incorporated monomer.	Taq DNA polymerase lacks a 3' to 5' exonuclease activity (proofreading activity). This is important for our studies since we do not want the enzyme to remove a base for which fluorescent signal was detected. If the enzyme used in single-molecule DNA sequencing possessed a 3' to 5' exonuclease activity, the enzyme would add another base to replace of the one that had been removed. This newly added base would produce a signature fluorescent signal that would suggest the presence of two identical bases in the template. This type of artifact could be detrimental to the technology. **P12,¶3**
104.(**previously presented**) The composition of claim 89,		

	wherein the polymerase lacks the ability to remove a previously incorporated monomer	Taq DNA polymerase lacks a 3' to 5' exonuclease activity (proofreading activity). This is important for our studies since we do not want the enzyme to remove a base for which fluorescent signal was detected. If the enzyme used in single-molecule DNA sequencing possessed a 3' to 5' exonuclease activity, the enzyme would add another base to replace of the one that had been removed. This newly added base would produce a signature fluorescent signal that would suggest the presence of two identical bases in the template. This type of artifact could be detrimental to the technology. **P12,¶3**
105.(**previously presented**) The composition of claim 79,		
	wherein the site is less than or equal to about 25Å from a tag on each incorporating monomer.	**Not specifically in the proposal. But the placement of the donor on the polymerase relative to the acceptor on incorporating monomer is not disclosed by either Korlach et al. or Schneider et al.**
106.(**previously presented**) The composition of claim 89,		
	wherein the site is less than or equal to about 25Å from a tag on each incorporating monomer.	**Not specifically in the proposal. But the placement of the donor on the polymerase relative to the acceptor on incorporating monomer is not disclosed by either Korlach et al. or Schneider et al.**
107.(**previously presented**) The composition of claim 13,		
	wherein a polymerase comprises any molecule or molecular assembly capable of polymerizing a set of monomers into a polymer having a predetermined sequence of monomers and a monomer comprises any molecule capable of being incorporated into a polymer having a predetermined sequence of monomers by a polymerase.	**Not in the proposal, but these definitions of polymerase and monomer are not disclosed in either Korlach et al. or Schneider et al.**